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Analysis and Application of Key Modeling Concepts Utilized in Predictive Microbiology for Food Processing

Darryl G. Black
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Darryl G. Black entitled "Analysis and Application of Key Modeling Concepts Utilized in Predictive Microbiology for Food Processing." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

P.M. Davidson, Major Professor

We have read this dissertation and recommend its acceptance:

John Mount, Federico Harte, X. Philip Ye

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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ANALYSIS AND APPLICATION OF KEY MODELING CONCEPTS UTILIZED IN PREDICTIVE MICROBIOLOGY FOR FOOD PROCESSING

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Darryl G. Black
May, 2008

Dedication

I would like dedicate this dissertation to my wife Tiffany, my daughter Ellen, my parents Grady and Emma, my brothers William and Ronnie, and in loving memory of my brother Robert.

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I would like to graciously thank Dr. Mike Davidson for his direction, support and patience on the work involved in this project. Without his insight and understanding, this accomplishment would not have been possible. I would also like to thank my committee members, Dr. John Mount, Dr. Federico Harte and Dr. Philip Ye for their invaluable help and guidance throughout my studies and research.

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Abstract

The use of modeling techniques for safety and risk prediction in the food supply is a common practice. Factors affecting microbial heat resistance include those inherent to the organism, environmental conditions and the intrinsic properties of the heating menstruum. Varying physiological states of microorganisms could affect the measured response and add uncertainty to results from predictive models. Inactivation tests were performed using *Escherichia coli* strain K12 and *E. coli* O157:H7 for various growth conditions: traditionally or statically grown cells, chemostat-grown cells, and chemostat-grown cells with buffered feed media. Heating menstruum was non-buffered 0.1% peptone, 0.1 M phosphate buffer (pH 7.0), a simulated beef broth (pH 5.9) and actual beef broth obtained from 93% lean ground beef. Thermal inactivation of the cells was carried out at 58, 59, 60, 61 and 62°C and recovery was on a non-selective tryptic soy agar. Chemostat cells were significantly less heat resistant than the traditional or buffered chemostat cells at 58°C. Shape response was also significantly different, with traditionally-grown cells exhibiting reducing thermal resistance over time and chemostat cells showing the opposite effect. Buffering the heating menstruum to ca. pH 7 for both traditionally-grown and chemostat cells resulted in inactivation curves which showed less variability or scatter of data points. Non log-linear regression analysis resulted in the most accurate fit in most cases. There were significant differences in thermal resistance when cells were thermally treated in either simulated or actual beef broth mixtures compared to laboratory diluent.

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PART ONE: REVIEW OF THE LITERATURE

Modeling concepts and equations for predictive modeling in food microbiology

Abstract

Modeling is a simulation technique that can take many forms depending on the scope and desired results from the object or phenomenon being modeled. While models can be qualitative, research scientists in the agricultural sciences are often more interested in the inference gained from data collected relative to an imposed treatment. Such information could be the reduction of a population of specific bacteria in a food product with the addition of a heat treatment. A model classification system has been proposed which gives a structured scheme composed of primary, secondary and tertiary models. Several models and mathematical functions have been utilized to enable scientists to relate such parameters as microbial growth, thermal death curves or inactivation due to antimicrobials. A model can be a linear representation of population density changes over time or consist of non-log-linear regression functions where there exists multiple independent variables which characterize different environmental conditions. A number of factors can affect microbial response during both growth and inactivation. Numerous researchers have conducted experiments which model the survival or inactivation of specific food pathogens in either real foods or laboratory media. Factors affecting microbial heat resistance include those inherent to the organism, environmental conditions and the intrinsic properties of the heating menstruum. The design of the thermal process controls the environmental and intrinsic conditions and can be influenced by experimental error and inadequate interpretation of results.

I. Definition of a model

Modeling can be described as a simulation technique capable of taking many forms depending on the scope and desired results from the object or phenomenon being modeled. Burnham and Anderson (*3*) stated that the goal of the researcher is to express information about a system or population of interest that exists in observed data in a 'model' that compresses and simplifies the observations to others. It is important to realize that models only offer an approximation to reality and there is typically not a 'true' model for any given system. Burnham and Anderson (*3*) recommend the concept of a global model which has many parameters, potentially covering all relevant effects, and that is based on the science of the system being modeled. This global model would be part of an *a priori* set of candidate models and should be tested for an acceptable fit to the observed data.

Many models associated with biological systems take the form of rate equations and are quantitative in nature. While models can also be qualitative, research scientists in the agricultural sciences are often more interested in the inference gained from data collected relative to an imposed treatment or condition, e.g., the reduction of a population of a specific bacteria in a food product with the addition of heat.

II. Microbial model classification

A model classification system for bacterial growth was proposed by Whiting and Buchanan (*41*) which gives a structured scheme composed of primary, secondary and

tertiary models. The standard bacterial growth curve, showing the change in microbial population over time, is considered a primary model. A secondary model describes the various phases of the primary model (lag time, growth phase, etc.) in relation to different environmental parameters such as temperature, pH, water activity or antimicrobial concentration. A tertiary model interfaces the secondary models with a user friendly interface in a computer program to produce primary model output (microbial population vs. time).

The mathematical functions discussed for microbial inactivation, growth and the growth/no-growth interface are examples of primary models. The result is the response of a microbial population with time for a given set of conditions. By definition, both D-value (time to reduce a population of microorganisms by 90% at a given temperature) and growth rate values are considered primary models. During the variation of specific conditions, such as temperature or pH, the response of the primary model will reveal the relative influence of the chosen independent conditions the microbial population experiences (*25*). An adequate primary model should describe the kinetics of the organisms' response using as few measured parameters as possible and result in an accurate portrayal of the stages in the process (*24*). It would not be inconceivable to have multiple primary models in a comprehensive analysis of a microbial response to various stimuli. The important step is to quantify the dependence of the primary model on the variation of input conditions.

A secondary model further defines the relationship between the input conditions and the response of the primary model by relating key parameters, such as specific

growth rate, to the variation of specific test conditions, such as temperature, pH and water activity. It is important that the secondary model includes all significant parameters important to the primary response of the microorganism (*24*). Secondary models can take the form of a polynomial relationship, an Arrhenius-type relationship or the z-value model (temperature change to cause a 1 log change in the D-value) used in classical microbial heat inactivation studies.

III. Interpretation of data and experimental error

Experimental error should always be considered when deriving a model, such as regression techniques used to estimate D and z-values from a collection of data for a specific microorganism. Datasets with values that appear extreme when compared to similar studies should always be closely analyzed if they are to be used as reference or validation data. The proper application of scientific method (in peer reviewed journals) is assumed to be the case, as well as the proper handling of replicates and samples. Many researchers include the methods used for proper reproduction of a given experiment, however it is not always practical for the researcher to detail each step. Proper evaluation of the methods used and robustness of data collected are key elements to modeling any experiment for the response of biological systems.

IV. Common mathematical functions used as primary models

Several models and mathematical functions have been utilized to enable scientists to relate such parameters as microbial growth or inactivation due to heat or antimicrobials. The model can be a linear representation of population density changes over time (i.e., first-order model) (*38*), which utilizes a rate constant to describe specific microbial response to environmental and intrinsic factors. Models may also consist of non-linear regression functions, such as Gompertz (*36*) or Weibull (*32, 40*), where there exists multiple independent variables which characterize specific conditions. Both linear and non-log-linear functions can be combined with polynomial regression of environmental conditions, such as temperature, pH and a_w, in order to generate predictive response curves.

Baranyi and Roberts (*1*) presented an explanation of various regression functions and differential equations used to model microbial growth. The authors cautioned against the use of the term 'model' for applications where regression techniques, such as Gompertz or polynomial functions, are used to simply fit experimental results. A robust mathematical model includes a set of defining hypotheses for the process and would likely use both regression functions and differential equations to analyze and enable prediction of response.

The instantaneous and specific growth rates of bacteria under defined conditions can be described by differential equations and are easily visualized as sloped lines on a semi-log plot of cell population versus time. The specific growth rate, $\mu(t)$, is defined by the relationship:

$$\mu(t) = \frac{dx(t)/dt}{x(t)}$$

where dx(t)/dt is the instantaneous rate or the absolute increase in cell concentration per unit time (positive for growth; negative for inactivation). The instantaneous rate is divided by the cell concentration at a given time point to define the specific rate. The value of the specific or instantaneous growth rates depend on the mathematical regression function used, which dictates the number and form of parameters optimized by the curve fitting process.

The Gompertz function is a non-linear, sigmoidal equation used to describe growth of microbial populations. The general form of the function used by some researchers (*17, 42*) is:

$$N(t) = A + C\, e^{-e^{-B(t-M)}}$$

Where *N(t)* is the population at time *t*, *A* is the initial population, *C* is the difference between initial and final population during stationary phase, *B* is the slope term indicating the maximum rate of growth at the inflection point of the sigmoidal curve, and *M* is the time when the inflection point occurs(or time to maximum growth rate).

Baranyi and Roberts (*1*) developed a more extensive sigmoidal model for microbial growth, which takes into consideration both inherent properties of the cell and the surrounding environment. The authors noted that using the Gompertz function

is a reasonable curve fitting procedure; however the method lacks physiological and environmental definition and should not be used to model bacterial numbers. The Baranyi-Roberts model contains three classes of variables: 1) intracellular conditions or a physiological state vector z(t), which takes into account certain biochemical concentrations such as biomass, enzymes or genetic material, 2) extracellular conditions influenced by bacterial metabolism (such as the production of lactic acid) which is designated as the category 1 external state vector c(t), and 3) extracellular conditions that are independent of the growing culture (such as environmental factors: temperature, pH and a_w) designated as the category 2 external state vector D(t). Due to the differential form of the equations used to characterize these variables, it is necessary to make several simplifying assumptions in order to obtain a useable model that fits the empirical data. A simplifying assumption that is common in many models is that the microbial population is homogeneous in the medium or food, showing no spatial variability in density or location. This assumption has no probability distribution for z(t) and any resulting error is referred to as homogeneity error. Another important simplification is that both the intra- and extra-cellular variables can be represented by relatively few variables, reducing the complexity of experiments. This introduces a completeness error which is directly related to how well the variables are characterized and measured. The kinetic form of the model can be expressed by the following differential equations:

$$dz(t)/dt = f(z(t), c(t); D(t))$$

$$dc(t)/dt = g(z(t), c(t); D(t))$$

The change in the physiological state vector z(t) per unit time t is described as a function of the instantaneous values of z(t) and c(t) given the external state vector D(t). Another function exists for c(t) per unit time. A common external condition described by D(t) is temperature, which is often described as steady state or constant during the process. The instantaneous growth rate, $\mu(t)$ is defined by another function of these vectors:

$$\mu(t) = \phi(z(t), c(t); D(t))$$

And the change in the concentration of the homogeneous population, x(t) can be described by:

$$dx(t)/dt = \mu(t)x(t)$$

The system of equations has a unique solution as long as the initial conditions are defined ($z(t_0) = z_0$; $c(t_0) = c_0$; $x(t_0) = x_0$) and the solution of cell concentration, as the natural logarithm, is in the form y = ln x(t):

$$y(t) = yo + \mu_{\max} A(t) - \frac{1}{m}\ln(1 + \frac{e^{m\mu_{\max} A(t)} - 1}{e^{m(y_{\max} - y_0)}})$$

Where y_{max} = ln x_{max} and $\mu_{\max}$ is defined as the maximum slope of instantaneous growth at the inflection point of the sigmoidal curve. The $\log_{10}$ denotation of *N(t)* is replaced

with *y(t)* to help denote the change to natural log (ln) transformation of the population value. The *A(t)* function represents a gradual time delay for growth and contains the physiological state vector, *z(t)*, and the external condition vectors, *c(t)* and *D(t)* are held constant. For a more detailed derivation of these terms, see (*1*).

A number of researchers have developed growth/no-growth models (*20, 24, 27, 34, 35*) to predict external conditions where actual growth would result. One goal of this work is the quantification or development of growth inhibition boundaries as defined in 'hurdle technology' (*21*). Ratkowsky and Ross (*35*) proposed a logistic regression model for growth versus no-growth that was based on a square root model by McMeekin et al. (*26*) where temperature, pH and water activity were considered. The square-root model proposed by McMeekin et al. was an extension of the Belehradek square-root model that originally included only temperature effects, and is given by the following:

$$\sqrt{k} = C\left(T - T\min\right) \times \sqrt{(pH - pH_{\min}) \times (a_W - a_{W\min})}$$

where T_{min}, pH_{min} and a_{Wmin} are the lower limiting values of temperature, pH and water activity that govern the growth/no-growth boundary and *C* is a regression constant. In this form, the equation acts as a secondary model which can be used to define the specific rate (*k*) for a primary model under the conditions chosen. Ratkowsky and Ross replaced the left-hand side ($\sqrt{k}$) with the 'logit p' expression used as a probability distribution function to represent the growth/no-growth boundary.

In the case of log-linear regression, the specific rate is a first-order function, resulting in a linear approximation to the measured response. The logarithmic order-of-death modeling equation (*33*) is:

$$\log_{10}(N(t)) = \log_{10}(N(0)) - \left(\frac{t}{D}\right)$$

where

$$D = \frac{2.3026}{k}$$

N(t) represents the population of surviving cells at time *t* of an inactivation process and *No* is the initial population. Population values are transformed into $\log_{10}$ values to enable proper scaling for plotting purposes to facilitate linear regression analysis. The first order rate constant, *k*, is the effective slope of the resulting population counts on a semi-log plot, with $\log_{10}$ population on the vertical axis and time *t* on the horizontal. This rate constant is related to *D* (or D-value) by conversion from natural log (*ln*) to $\log_{10}$. D-value then represents the inverse slope of the inactivation curve, or the time to realize a 1 $\log_{10}$ (90%) reduction in the observed population given the conditions of the process (temperature).

Alternatives to the log-linear order-of-death are supported in the literature (*22, 32, 40*). Justification by researchers for non-log-linear functions is the frequency of non-log-linear responses in the semi-logarithmic survival curves of many microbial spores

and vegetative cells. The simplified form of the non-log-linear Weibull probability density function which is applied to microbial inactivation is:

$$\log_{10}(N(t)) = \log_{10}(N(0)) - \left(\frac{t}{\delta}\right)^{p}$$

Where *N(t)* is the number of microorganisms at time t , *N(0)* is the initial number of microorganisms present. The distinct parameters of the function are δ (the characteristic time or hazard function) and *p* (the shape factor). The δ term is similar to the first order rate constant (*k*) and gives an overall slope or rate of change to the curve. The *p* term allows the function to take on a non-log-linear shape, where a value of $p<1$ results in a concave downward shape and $p>1$ is concave upward.

V. Common secondary models

One of the most common secondary modeling techniques is the use of polynomial regression to describe the specific rate (growth or death) in terms of parameters such as temperature, pH or water activity using a multiple order polynomial equation (*16, 17, 24*). A general form of a polynomial equation was presented by Gibson, et.al (*17*) to describe growth rate at various pH, temperature and water activity combinations:

$$y = a + b_1 s + b_2 t + b_3 p + b_4 s^2 + b_5 t^2 + b_6 p^2 + b_7 st + b_8 sp + b_9 tp + e$$

Where *y* is the response variable, such as *B* or *M* in the Gompertz equation, s represents NaCl percentage (thus water activity), *t* represents temperature, *p* represents pH, and e is the random error associated with the regression (having a mean of zero and some observed variance). The ease of use, availability of statistical packages, applicability to multiple regression techniques, and the fact the equation can be used to combine an almost unlimited combination of environmental conditions, makes the use of polynomial secondary models very appealing. The polynomial equation is the most common form used in secondary models for growth or inactivation.

There is some danger in depending solely on polynomial equations as adequate secondary models, especially if employed outside the range of experimental data used as a basis for the overall model (*24*). Another point to consider is that many of the coefficients used to obtain a best-fit of the data do not have true biological interpretations. This fact is readily seen in the example equation above, which uses nine coefficients to describe three environmental conditions. Another danger associated with higher order polynomials, containing squared and cubed terms, is the fact these equations may fit the observed data adequately, but outside the data range the predictions could asymptotically or exponentially exaggerate predictions beyond the limits of experimentally observed values. The use of constrained linear polynomial models is highly recommended in such cases and is evident in several tertiary models (computer models which only allow a prescribed range of input values). Even with these constraints, some researchers warn that polynomials should only be applied under strict

conditions where the quantity and quality of experimental data support the technique (*2*).

Secondary models based on the Arrhenius equation are derived from the energy of activation associated with enzyme kinetics. These models are primarily used for growth kinetics and in their purest form only consider temperature as an independent condition. Davey and Daughtry (*7*) discussed the use of a modified Arrhenius equation to model the temperature dependence of growth kinetics for foodborne bacteria. The authors cited the widely used empirical form of the Arrhenius equation:

$$k = A\, e^{\frac{E}{RT}}$$

where *E* is the activation energy in J/mole, *A* is the frequency in time^{-1}, *R* is the universal gas constant, *T* is temperature in degrees Kelvin and *k* is the specific rate of reaction. McKellar and Lu (*24*) took the natural logarithm of both sides of this equation which resulted in the following:

$$\ln(k) = \ln(A) \times \frac{E}{RT}$$

If ln(*k*) is plotted against (1/*T*), the temperature range that promotes microbial growth can be extrapolated from the straight line portion of the graph and the activation energy (*E*) estimated by solving the above equation over that range. This activation energy value can then be used to characterize the microorganism under the conditions tested and used to attain specific growth rates for all temperature values within that

range. Modifications made to the model by Daughtry et al. (*4*) resulted in the following form of the equation:

$$\ln(k) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2}$$

where C_0-C_2 are coefficients that can be estimated using regression and the additional $1/T^2$ term is used to account for curvature found using the traditional form of the Arrhenius relationship. While this relationship resulted in a more accurate fit to experimental results, it only considered the secondary relationship with temperature. Additional modifications by Davey (*5*) considered other conditions in an Arrhenius type approach. Adding the affects of water activity, the author developed the following equation:

$$\ln(k) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} + C_3 a_W + C_4 a_W^2$$

where C_0-C_4 are coefficients determined from regression. In later work (*6*), Davey developed a similar relationship for modeling the addition of pH and subsequently, Davey and Daughtry (*7*) added both water activity and pH to form the modified Arrhenius equation:

$$\ln(k) = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} + C_3 S + C_4 S^2 + C_5 pH + C_6 pH^2$$

where *S* is salt concentration given as %w/v, representing changes in water activity. Daughtry et. al (*4*) further developed a modified form of the Arrhenius relationship, resulting in a more linear response function:

$$\ln(k) = C_0 + \frac{C_1}{T} + C_2 \ln(T).$$

The natural log transformation of *T* results in a larger multiplier than the inverse of T^2 and changes the magnitude of the C_2 coefficient. A similar relationship was developed by the researchers for determining lag time as a function of temperature. Both equations were shown to accurately fit experimental results from a large collection of independent studies, using just temperature as the independent variable.

A key secondary model for thermal inactivation is the z-value. The classical model of thermal inactivation originally developed for the canning industry (*33, 38*) that utilizes the D-value was further defined by this basic parameter. Both D and z-values have evolved from their original definitions where they were primarily used to describe moist heating conditions for the inactivation of *Clostridium botulinum* spores (*33*). The definition of z-value is the number of degrees (either ^{o}F or ^{o}C) required to cause a one log cycle (90%) change in the log of the D-value. This parameter is a useful tool that is readily applied to the reduction of overall process time by increasing process temperature and has been well established for multiple microorganisms in the literature. When applying the z-value or D-value models to alternative processes, such as non-thermal applications, the researcher should describe the experimental

conditions, the mathematical techniques used and disclose all assumptions to support the conceptual use of the model (*33*).

VI. Factors affecting microbial heat inactivation and model selection

The production of safe foods requires that pathogenic microorganisms are inactivated or their growth controlled prior to consumption. A common means of inactivation used in food processing is the application of heat. The mode of action for thermal inactivation is the denaturation of nucleic acids, structural proteins, and enzymes (*12*). DNA damage is considered one of the key lethal events in heated cells, leading to the inability of the microorganism to reproduce. A number of factors can affect microbial response during thermal inactivation. Factors affecting microbial heat resistance include those inherent to the organism, environmental conditions and the intrinsic properties of the heating menstruum (*9*). Environmental conditions can be both physical and chemical. The design of the thermal process controls the environmental and intrinsic conditions and can be influenced by experimental error and inadequate interpretation of results.

VII. Microbial and recovery factors influence apparent heat resistance

Variation in the resistance to heat occurs among types of microorganisms (i.e., bacteria, yeasts, molds), between vegetative cells and spores, and among genus, species and even strains of microorganisms. In designing thermal inactivation experiments, the researcher should select the appropriate test microorganism for the purpose of the

thermal process. For example, pasteurization treatments require vegetative cells while sterilization requires the use of bacterial spores. There are a great many factors involved in the selection of the target microorganism and the recovery of viable cells. Failure to use appropriate conditions could lead to thermal process failure.

While it is not good practice to use a single strain as a standard for all strains of that microorganism, there are some issues in heat inactivation studies when mixtures of strains or "cocktails" of multiple strains are used. Reviews on the heat resistance of *Salmonella* spp., *Listeria monocytogenes* and *E. coli* O157:H7 by O'Bryan et al. (*31*) and Doyle and Mazzotta (*10*) illustrate the tendency of many researchers to combine various strain mixtures when providing thermal inactivation data on actual food products. The problem with using strain mixtures for heat resistance studies is illustrated for studies using *S*. Senftenberg (775W) which has a much higher heat resistance than other *Salmonella* strains. The heat inactivation of strain mixtures containing this particular organism resulted in more rapid decline of the less resistant strains at the beginning of the process followed by a more gradual reduction of *S.* Senftenberg at later time points. Whiting and Buchanan (*42*) described the existence of more heat resistant sub-populations of bacterial spores that were observed by various researchers. The authors described a combination of first-order processes for the rapid inactivation of less heat-resistant spores followed by a more gradual reduction of the more resistant sub-population, resulting in a bi-phasic inactivation curve.

Growth conditions used to prepare a microorganism for heat resistance studies can greatly influence the inherent physiological properties (stress adaptation) of the

microorganism and thus influence heat resistance (*14, 19, 23*). Edelson-Mammel et al. (*14*) studied the heat resistance of 13 individual strains of *L. monocytogenes* grown at various pHs. The pH differences were achieved by growth in either tryptic soy broth with glucose (TSB + G; growth pH 4.7) or tryptic soy broth without glucose (TSB - G; growth pH 6.7). The majority of combinations tested (14 of 26) showed the acid-induced cells to have significantly higher heat resistance. The authors concluded that the variation of pH of the growth media could either increase or decrease the thermal resistance of *L. monocytogenes*, depending upon the strain. A study on acid-stressed *Shigella flexneri* (*39*) indicated increased resistance to extreme acid and temperature conditions. Acid adapted cells (1% glucose added to TSB) had recovery rates after 48h at 48°C of 3.4 log compared to <1 log CFU/ml for non-adapted cells. Manas et al. (*23*) studied the heat resistance of different serotypes of *Salmonella* relative to pH and growth temperature. The researchers reported a 4-fold increase in D-value for *Salmonella* Typhimurium grown at 37°C compared to growth at 10°C. A response surface regression model was developed from the data to calculate the relative influence of growth temperature versus pH.

The heat resistance of facultatively anaerobic microorganisms can also vary relative to their gaseous growth environment (either aerobic or anaerobic). Murano et al. (*28*) tested the effect of growing *E. coli* O157:H7 aerobically versus anaerobically on their subsequent heat resistance and found that anaerobically grown cells were more heat tolerant (under non-heat shock conditions). D_{55C} values for aerobically versus anaerobically grown cells were 8.0 versus 11.1 min, respectively. The researchers also

tested the effect of heat shock prior to the thermal process. Results indicated increased heat resistance of *E. coli* O157:H7 when subjected to sub-lethal heat shock at 42°C for 5 minutes. Their results show 2.5 log CFU/ml higher numbers of survivors after a 20 min process at 55°C for heat shocked (42°C for 5 min) compared to non heat shocked cells. The differences discussed above for aerobically vs. anaerobically grown cells were overshadowed by heat shocking at 42°C for 5 min, with D_{55C} values of 16.8 and 16.7 min. Protein analysis (using Western Blot) for both heat shocked and anaerobically grown cells showed similar levels of a protein found to be immunologically similar to a δ^{32} sub-unit of RNA polymerase (thought to provide heat shock protection). These proteins were not present in aerobically grown, non heat-stressed cells. This research helps illustrate the relative effects of different growth conditions on the thermal inactivation properties of an organism. Alternatively, Kaur and others (*19*) found no significant heat resistance effect from elevated growth temperature (40°C), heat-shock at sub-lethal temperatures (42, 45, 48 and 50°C), or from variable heating rates (1°C to 23°C/min). The researchers did notice a dramatic increase in heat resistance for late stationary phase cells compared with log growth phase cells. Their studies also showed significant increases in heat resistance with decreasing water activity.

Generally, when using pure cultures, a non-selective microbiological medium is used for recovery of surviving microorganisms in a thermal resistance experiment. If background microflora are present, a selective recovery medium may be needed for recovery of surviving cells. Selective media contain chemicals which help isolate the microorganism of interest by inhibiting other groups or types of microorganisms. An

example would be the use of the selective agent, bile salts, to inhibit the growth of gram positive microorganisms but allow the growth of coliform bacteria, including *E. coli*. However, when those selective agents are used, injured target microorganisms may not be able to grow in the recovery medium, i.e., due to their injured state, heat damaged microorganisms may be inhibited by the selective agents to which they are normally resistant. Researchers have utilized numerous techniques, which incorporate various additives, for the recovery of injured microorganisms at the later points of inactivation processes. Duffy et al. (*13*) studied differences in thermal tolerance of various strains of *E. coli* O157:H7 in salami. The researchers compared the use of a non-selective agar (trypticase soy agar, TSA) overlay for the selective sorbitol MacConkey agar (SMAC) and compared it with the SMAC only. Recovery of cells heated to 50 and 55°C showed sub-lethal injury ranging from 72 to 88% and 64 to 97%, respectively for the two methods. Results indicated the use of selective recovery media only could underestimate D-values for the specified process.

Another factor that may influence recovery of heat treated microorganisms is clumping. Researchers have discussed the concept of cell/spore clumping in food products during processing as a reason for a flattening or tailing of the survivor curve (*8, 9, 37*). Cells or spores located near the center of the proposed clump do not receive the same thermal treatment and thereby survive the heating process longer. The clump theory offers considerable challenge to experimental validation and must be approximated with theoretical models.

VIII. Food-related factors influencing apparent heat resistance of microorganisms

Researchers conduct experiments to model the survival or inactivation of specific foodborne pathogens in either real foods or laboratory media. Some of the common intrinsic factors evaluated for their influence on thermal inactivation are pH and water activity. These and other intrinsic properties of the food should be considered when designing thermal inactivation experiments.

Stringer et al. (*37*) compiled inactivation data for *E. coli* O157:H7 heated in different menstrua such as broth and buffers, apple juice, poultry meat and red meat. Results for the combination of all menstrua (z-value of 7.6°C and D_{60C} = 1.6 min) showed considerable variability (scatter) with an R^2 = 0.54. The results for broth and buffers alone showed even less correlation (R^2 = 0.31), which could indicate a wide range of methods (or formulations) used. The overall linear fit and effective slope (denoting z-value) changed as the heating menstruum became more homogeneous. The results for apple juice (z-value of 7.4C and D_{60C} = 0.8 min) showed considerably higher correlation with an R^2 of 0.75. The D-value at 60°C for apple juice was half that of the combined data (0.8 min for apple juice compared to 1.6 min for all menstrua). A significant shift in both linear fit and z-value (slope) was seen in meat products. The results for all meat together showed the highest correlation with an R^2 of 0.85 (z-value of 5.5°C and D_{60C} = 1.8 min). Though the D_{60C} value for meat products was similar to all menstrua, the fact the z-value was more than 2°C less resulted in considerable divergence of D-values on either side of this temperature. Data compiled on thermal inactivation of *Salmonella* show variation depending on the meat type. Using the same mixture of strains, similar

thermal resistance measurement techniques and the same recovery media, Murphy et al. (*29, 30*) found significantly different D-values for chicken breast meat vs. thigh and leg meat, i.e., D_{55C} of 24.07 or 43.76 min, respectively. The effect was less pronounced as temperatures increased. The z-values reported for each meat type were nearly 1°C different (6.26°C for breast meat and 5.34°C for thigh/leg meat).

The effect of free water (higher a_w) in the heating menstruum can be seen in research by Doyle and Mazzotta (*10*) regarding the inactivation of *Salmonella* spp. in molten chocolate. D_{71C} values in chocolate for various strains ranged from approximately 210 to 1200 min. Reported z-values were relatively high (19°C) compared with other food types. The authors suspected the extremely low water activity (0.75 to 0.84) contributed to the high D-values and they noted large changes in D-value relative to added moisture (D_{71C}= 1200 min with 0% added moisture versus 240 min with 2% added moisture). Doyle et al. (*11*) evaluated data from various thermal inactivation studies in culture media with varying sugar and salt concentrations. Studies conducted with increasing sugar concentration (decreasing water activity) showed significant increases in D-values (D_{60C}=2.0 min at a_W of 0.98 versus 8.4 min at a_W of 0.90). Similar trends were seen in salt solutions, with a D_{60C}=0.5 min at 0.5 M NaCl versus 3.3 min at 1.5 M NaCl.

Some Intrinsic factors have the potential of providing micro-environments and protective components that could result in tailing because of delays in heat penetration. For example, Geeraerd et al. (*15*) described how proteins and fats can add to the thermal tolerance of microorganisms during thermal processing. Proteins could help

prevent the loss of solutes, stabilize the cell membrane and provide a buffering effect against lower pH conditions. Fat molecules could create a reduction of water activity (reducing thermal delivery) due to solubility changes at increasing temperatures. Juneja and others (*18*) studied the effect of different fat levels on the inactivation of *Salmonella* in poultry products. Both a range of fat levels (1-12%) and process temperatures (58-65°C) were tested and the most significant increases in heat resistance (D-value increase of 23%) were found for 12% fat products at the lowest process temperature.

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Use of modeling to enhance the microbiological safety of the food system

Abstract

The use of modeling techniques for safety and risk prediction in the food supply is strongly supported by the incidence of foodborne outbreaks worldwide. In microbiological food safety, important parameters include incidence, microbial growth, microbial inactivation or survival and dose response of the host. The goals of the modeler should include: 1) identifying possible inputs and outputs for a particular concern and 2) choosing factors for integration into a larger, overall model. A case study of ground beef from the farm to the consumer reviews multiple points of concern where relevant parameters for *Escherichia coli* O157:H7 can be modeled. The prevalence and concentration of *E. coli* O157:H7 in cattle have been confirmed and studies have explored various feeding regimens and hide-cleaning methods for controlling the pathogen prior to slaughter. There is significant impact of incidence and cross-contamination during slaughter operations. Intervention methods, such as washing and heat treatments, and proper carcass handling may reduce the contamination level. Microbial growth in meat products can be extensive, given the possibility of temperature abuse during transportation and storage. Dose-response models for susceptible consumers provide a safety perspective for quantitative microbial models. Growth prediction and lethality models, available on-line, are valuable tools for both researchers and processors. The goal of the processor should be the “highest reduction possible” (to meet adequate safety requirements) prior to the preparation of raw

product. The researcher must continually question the use of a model and the availability of data to validate any conclusions.

I. Introduction

Modeling can be described as a simulation technique capable of taking many forms depending on the scope and desired results from the object or phenomenon being modeled. Burnham and Anderson (*10*) stated that the goal of the researcher is to express information about a system or population of interest that exists in observed data in a 'model' that compresses and simplifies the observations to others. An important concept in modeling is that models only offer an approximation to reality and there is typically not a 'true' model for any given system. Burnham and Anderson (*10*) recommend the concept of a global model which has many parameters, potentially covering all relevant effects, and that is based on the science of the system being modeled. This global model would be part of an *a priori* set of candidate models and should be tested for an acceptable fit to the observed data.

Many models associated with biological systems take the form of rate equations and are quantitative in nature. While models can also be qualitative, research scientists in the agricultural sciences are often more interested in the inference gained from data collected relative to an imposed treatment or condition, for example, the growth of spoilage organisms under certain environmental conditions or the reduction of a specific microbial pathogen in a food product with the addition of a heat treatment.

II. Microbial Concerns in the Food System

Knowledge of the existence of particular microbial pathogens and identification of their association with certain foods has changed radically. For example, a general guide for food processors published in 1975 by the National Research Council (Washington, D.C.) (*15*), cited only a handful of the currently recognized foodborne pathogens (*69*). Notably, there was a single pathogenic strain of *Escherichia coli* (Enteropathogenic) and many species of currently recognized pathogens, such as *Campylobacter jejuni,* were absent. *Salmonella* was listed as the major concern in red meats while there was no mention of *E. coli*. With advances in molecular detection and typing, as well as an overall increased knowledge of the microbial agents responsible for food-related illness, much has changed over the past 30 years (*69*). This level of differentiation of microorganisms and their association with specific foods is necessary in order to apply an adequate level of processing for specific products and to have awareness of the possible severity associated with exposure to particular foodborne pathogens.

Research on the influence of intrinsic, extrinsic, and processing factors on growth and survival of foodborne pathogens has contributed to a current bank of information (*35*). While the basic thermal process to eliminate *Clostridium botulinum* from low-acid canned foods has been known since the 1930s, information on heat resistance and other stresses (for example, pH, and water activity) for many foodborne pathogens has taken longer to gather. Now, factors such as atmosphere, temperature, pH, water activity, oxidation/reduction potential, and combinations thereof along with the

presence of antimicrobials and stress adaptation are all known to play a role in determining which microbial pathogen is of most concern in a particular product or process.

Other major changes that have occurred associated with food safety are the expectations of safety by the consumer, the types of products available, and the processes used to control microorganisms in foods. In the latter part of the 20th century and into the 21st century, there has been an increased expectation by consumers that the responsibility for microbiological safety lies with the food processor and food producers. Prior to this change in attitudes, using proper handling techniques (such as, avoiding cross contamination) and adequate cooking, to produce safe products were considered to be the responsibility of the consumer. It is now expected that producers/processors should produce raw products with few or no microbial pathogens present. For example, lettuce or spinach that is contaminated in the field or in a further processing plant with *E. coli* O157:H7 (*2*) is a hazard for the consumer because it undergoes no process that is lethal to the microorganism. With trends toward fresh and organic foods, greater understanding is needed of contamination routes and the influence of handling and processing steps on microbial growth and survival.

III. Modeling Food Safety

A comprehensive review by McMeekin and Ross (*49*) offers strong support for the development of food safety models through the use of predictive microbiology

techniques. The authors point to changes in the food industry and consumer preferences since the mid-1970s that have resulted in major developments in food microbiology. Many researchers have discussed the importance of modeling growth, survival, and inactivation of pathogens, thereby developing the concept of "predictive microbiology" in regard to food safety (*5, 50, 60, 61, 73*). Other researchers have utilized more comprehensive techniques, such as Monte Carlo simulation, to develop quantitative risk assessment models to describe large processes with multiple interaction steps including growth and inactivation models (*12, 13, 21*), Monte Carlo simulation utilizes probability distribution functions, such as the normal distribution, as input variables in a set of governing equations in order to calculate likely distributions of defined outputs. A random number generator is used to populate the input function in a series of iterations or trials that represent different values of the input (incoming concentration of bacteria of an infected beef trimming) relative to the desired output (prevalence and concentration of the bacteria in the finished product).

A model classification system was proposed by Whiting and Buchanan (*72*) which gives a structure scheme composed of primary, secondary, and tertiary models. The standard bacterial growth curve, showing the change in microbial numbers over time, is considered a primary model. A secondary model describes the various phases of the primary model (lag time, growth phase, and so on) in relation to different environmental parameters such as temperature, pH, water activity, or antimicrobial concentration. A tertiary model interfaces the secondary models with a user-friendly

interface in a computer program to produce primary model-predicted output (microbial density vs. time).

The production, manufacturing, and consumption of foods throughout the world offer a tremendous challenge to scientists wishing to develop an overarching risk assessment model that helps ensure microbiological safety of foods to the consumer. To develop such an overarching risk assessment model requires that the "farm to table" food system be sub-divided into inputs that influence the overall microbiological safety of a food product. The important input parameters to model should include the incidence and concentration of foodborne pathogens on raw products, microbial growth during production, processing, transportation and storage, microbial inactivation/survival during processing or in the home, cross contamination at the production, processing and consumer stages, and, finally, dose response of the host. Some of these inputs have been modeled in detail while others are much less well described. The objective of this discussion is to review the state of models for each of these inputs and show how the models might be used to develop an overall risk assessment using a case study.

IV. Modeling Incidence of Foodborne Pathogens

The incidence (prevalence) of a specific microbial pathogen, for a given reservoir, is generally described as a percentage of occurrence relative to the overall population of interest (*12, 13, 21, 39*). Researchers have used a beta probability distribution

(sometimes referred to as a beta-Poisson) to describe the prevalence, or the odds, of infection or contamination. It is possible to combine the overall prevalence of occurrence with concentration studies of infected populations. Various examples of the use of probability distributions for prevalence and concentration are discussed below in the case study.

V. Modeling Growth

One of the two most studied areas of modeling of microbiological food safety involves microbial growth. Sigmoidal shape functions have been used effectively to model the lag, exponential growth, and stationary phases associated with microbial growth. Schaffner and Labuzza (*61*) described the use of the Gompertz function by various researchers and its advantages over bi-phasic approaches which separate the lag section from the period of exponential growth. Baranyi and Roberts (*5*) presented an explanation of various regression functions and differential equations used to model microbial growth. The authors cautioned against the use of the term ”model” for applications where regression techniques, such as Gompertz or polynomial functions, are used as ”good fits” to experimental results. The authors proposed a sigmoidal function which takes into consideration the physiological state of the microbial population at the time of growth. A robust mathematical model would include a set of defining hypotheses of the process and would likely use both regression functions and differential equations to analyze and enable prediction of response.

Likely the most useful methods for assessing growth potential of a pathogenic microorganism are growth prediction models. These models are extremely useful in modeling temperature abuse during processing or retail storage. For example, Cassin et al. (*12, 13*) used the tertiary modeling program Growth Predictor (www.ifr.ac.uk/Safety/GrowthPredictor/default.html; (*36*), formerly known as the Food MicroModel, to determine a range of specific growth rate and lag times between 10 to 15°C, pH 5.1 to 6.1, and water activity from 0.99 to 1.0 to estimate potential growth of *E. coli* O157:H7 on ground beef. These values were used as inputs to calculate the growth level in log CFU/g using the Baranyi model (*6*). While these are only estimates, because some models are derived from studies with microbiological media, they can give an idea of the risks involved during storage of a food product. Another tertiary model, the Pathogen Modeling Program (PMP; www.arserrc.gov/mfs/pathogen.html; USDA Agricultural Research Service (*70*) allows the user to select a number of different pathogenic and spoilage microorganisms and predict events such as aerobic growth, anaerobic growth, cooling survival, and various means of inactivation. Several antimicrobial compounds are included for individual microorganisms under both growth and inactivation conditions. Similar to the Growth Predictor model, the primary extrinsic factors necessary are temperature, pH, water activity, and specific antimicrobial compounds and their concentration. The growth models referenced in the PMP primarily use the Gompertz function along with various secondary models to predict growth. Much of the data used to generate the model are based on microbial growth in microbiological media rather than in foods. A third important resource is a database of

microbiological research, known as Combase (wyndmoor.arserrc.gov/combase/default.aspx; (*24*). This combined relational database is a collaboration of the Institute of Food Research (Norwich, UK), the Food Standards Agency (UK), and the USDA Agricultural Research Service's Eastern Regional Research Center (Wyndmoor, PA). ComBase contains thousands of data sets of microbial responses in both media and food environments for various extrinsic conditions. These data sets provide the basis of information for both the Growth Predictor and PMP. The report structure is displayed with drop-down fields for selection of microorganism, media, and growth conditions. Search criteria include range fields for temperature, pH, water activity, and anti-microbial compounds.

The importance of competition should be considered when developing growth models. Powell and co-workers (*56*) described a number of studies in which competitive inhibition and a resulting suppression in growth of the pathogen resulted when in co-culture with normal spoilage microflora. They developed a model for competition relative to *E. coli* O157:H7 in ground beef fabrication. Other models have been developed that provide a mathematical means of predicting survival of particular pathogens when exposed to various lactic acid bacteria (*37, 57*).

VI. Modeling Cross-Contamination

The concept of accurately modeling the cross-contamination of a pathogen population from one food component (or animal) to another is challenging. Such

calculations rely on the interaction of many variables. Researchers often apply simple ratios or percentage estimates to define the probability and extent of cross-contamination at specific points in a system or model (*13, 14, 64*). Luber et al. (*47*) studied the cross contamination of kitchen surfaces and utensils by raw poultry contaminated with *Campylobacter jejuni.* Lekroengsin et al. (*46*) developed correlation equations for contamination by *Listeria* spp. between poultry products and environmental surfaces during processing. More modeling techniques and raw data, which realistically predict the effects of cross-contamination during processing, are needed.

VII. Modeling Inactivation or Inhibition

The second of the most studied areas of modeling of microbiological food safety involves microbial inactivation/survival and inhibition. Several models and mathematical functions have been utilized to enable scientists to relate such parameters as microbial inactivation due to heat or other preservation treatments and inactivation or inhibition by antimicrobial food preservatives. A model may be a linear representation of population density changes over time, such as the first-order model used by Stumbo (*67*), which utilizes a rate constant to describe log-linear microbial inactivation over time at a given temperature. Models may also consist of non-log-linear regression functions, such as Weibull (*52, 71*), where there exist multiple independent parameters which characterize specific shape and magnitude of the primary population response relative

to time. Both linear and nonlinear primary functions can be combined with secondary models, such as polynomial regression functions of extrinsic conditions such as temperature, pH and water activity (a_w), in order to predict the primary model parameters for predictive applications.

Adaptation response of individual microorganisms may influence responses to stresses during processing (*1, 18, 38*). Abee and Wouters (*1*) noted the inactivation of the *rpo*S regulon (σ^{38}), which controls the expression of more than 35 genes in *E. coli,* renders the cells sensitive to a number of stresses commonly associated with minimal processing, such as presence of acid, heat, alcohol, or oxidative conditions. An understanding of the conditions that trigger *rpo*S to induce stress response in the organism is needed to determine the extent of stress adaptation and the subsequent levels of stress necessary to inactivate the microorganisms. Experiments which vary such stresses and develop the appropriate primary and secondary relational models become valuable sources of information for both modelers and those wishing to simply understand the sensitivity response to a particular system.

VIII. Modeling Infectious Dose - Concerns Related to the Host

To be useful in risk assessment, models need to be developed that predict the time necessary under specified conditions to produce an infectious dose of a foodborne pathogen. This requires knowledge of pathogenicity and factors affecting virulence.

Additionally, whether a microorganism causes an infection or intoxication is also important.

Intoxication requires that the microorganism produce a toxin in the food product. Many microbial models predict time-to-toxicity or predict the population level necessary for toxin production by toxigenic pathogens (*62, 74*). The foodborne pathogens responsible for production of toxins in foods include *Clostridium botulinum*, *Staphylococcus aureus,* and *Bacillus cereus.* The latter includes strains capable of both infection and intoxication. Intoxications by foodborne pathogenic bacteria generally require high numbers of microorganisms to produce sufficient toxin in food prior to consumption to induce illness. Understanding and effectively modeling the conditions necessary for toxin production is crucial. In the case of the heat-stable toxins of *S. aureus* produced prior to a final sterilization step, the organism would likely not be detected, but the toxin could still be biologically active (*16*).

An infection denotes the ingestion of a foodborne pathogen with subsequent attachment, invasion or toxin production by the microorganism in the gastrointestinal tract. For those foodborne pathogens that cause infections, predicting the time to produce an infectious dose may be more complicated. Important factors that need to be considered are dose response, pathogenicity, and virulence factors. Quantifiable factors may be the number of organisms relative to the time symptoms appear or the number of individuals of a given age or condition that become ill. Strachan et al. (*66*) used several single-hit models where the probability of illness from a single organism was determined. A common probability function used in these models is the exponential

Poisson distribution, which is often used in rare event analysis. The basis of this distribution is a binomial likelihood function centered on the mean number of rare occurrences, regardless of the population of trials. A two-parameter exponential distribution, known as a beta-Poisson function, is recommended by these researchers and others (*68*). This function relates the outcome of the event to the total number of trials and was found to fit experimental and outbreak data more precisely than the standard Poisson model. The beta-Poisson would be a logical extension of population models in order to determine the probability of illness for a given exposure. An example of the use of a beta-Poisson dose response model was given by Strachan et al. (2005) for an outbreak of *E. coli* O157 in a primary school in Scotland. Cheese made from unpasteurized goats' milk was consumed by 28 children with no product remaining for analysis and initial dose determination. By inputting the number of affected and asymptomatic individuals into the model, the likely dose was calculated to be 10^4cells.

IX. Deterministic or Probabilistic

It is convenient to think of point estimates from a mathematical function for population prediction as definitive or deterministic, depending on the robustness of the model and extent of the underlying dataset. The more likely case, as pointed out in the literature (*49, 53-55*), is that variability in population response is a more stochastic or random process when describing the results from a given model. Experimental variation, such as sampling or measurement error, and the inherent differences in

physiological response of individual cells are more accurately described by specific probability distributions of population density at a process time point.

McMeekin and Ross (*49*) provide an insightful review of concerns and limitations in predictive microbiology from the standpoint of microbial adaptation to changing environmental factors. The authors note that when one considers their small size, the ability to disperse easily, tolerate extreme conditions, show physiological diversity, and readily exchange genetic material, it is not surprising that many microorganisms have adapted to even the most extreme habitats on the planet. It is generally accepted that while large populations of microorganisms obey the rules of taxonomy, individual cells may show levels of variability in response that are the result of mutations or fluctuating local environments. The authors cite work by Bridson and Gould (*9*) where the distinction between quantal and classical microbiology can be illustrated in different sections of the growth curve. Large populations are characterized in "classical microbiology" and more adequately describe the exponential and stationary phases, whereas quantal (more probabilistic) traits are seen in the lag phase and the latter portions of the death phase. Adapted microorganisms display the ability to thrive in their historical environment and show sufficient survival, through heterogeneity, to more adverse conditions. The danger in food processing depends on the ability of these cells to retain viability and reproduce from relatively low numbers, after some period of physiological adjustment or recovery.

The work of Pin and Baranyi (*53*) on kinetic responses of single-cell vs. multiple-cell inocula illustrates the stochastic behavior of small numbers of microorganisms

under growth conditions. As the number of cells approaches one, variance of the lag time greatly increases. The lag responses were given by frequency histograms, which at higher numbers of inocula resembled a normal distribution with less variance. Under optimal growth conditions, the maximum number of cells used in the inoculum (100 for this study) converged on a lower lag time value with little variance.

X. Case Study: *E. coli* O157:H7 in Ground Beef

The key to using modeling techniques to improve overall safety in the handling and processing of foods is defining the parts of a process which can be qualitatively and quantitatively monitored for stepwise analysis. The goals of the modeler should include: 1) identifying possible inputs and outputs for development of models for home and industry clients for a particular concern and 2) choosing factors to be integrated into a larger, overall model (i.e., one that encompasses many sources of contamination). Simplifying assumptions and boundary conditions must be included in the definition of each input or output and are often necessary for model solutions. One such condition might be the assumption that a contaminant is homogeneously mixed in the product thereby minimizing the mathematical complexity and randomness of spatial variation within a particular food. Another important factor is to maintain unit distinction through a process and make the necessary calculations when transfers are made. An example would be the conversion of surface contamination by a microorganism in CFU/cm^2 to CFU/g following a meat grinding process. This requires another assumption of relative

surface area to weight of the contaminated portion and the conversion usually includes the assumption of homogeneity after grinding.

To better understand and determine appropriate inputs/outputs and the simplifying assumptions necessary for their inclusion, it is convenient to follow a generalized process and review some of the research that has been conducted for a particular food. The transmission of *E. coli* O157:H7 from the farm to the consumer in ground beef has been chosen as a case study. Some information on this case study has been reported previously (*12, 13, 21*). A recent review on the use of quantitative risk assessment modeling for *E. coli* O157:H7 on beef is available (*19*). Figure 1 shows an example of generalized flow for ground beef processing and its use by the consumer. (All figures and tables appear in the appendices.) There are numerous steps from the live animal to the consumer and those points which are key to determining the safety of the final product will be highlighted.

XI. Live Animal

The first input to be considered in determining the microbiological safety of ground beef, as to *E. coli* O157:H7, is the live animal. The prevalence and concentration of *E. coli* O157:H7 in cattle have been used previously as both inputs and outputs in risk assessment models (*12, 21, 39*). Cassin et al. collected information on the incidence of *E. coli* O157:H7 on cattle hides and in feces samples from a number of other studies that had sampled for various herd types and animal ages. They used a beta probability distribution (a two point parameter continuous function defined on the interval [0,1]) to

estimate prevalence values or the odds of infection for selected herds, where 0 to 3.1% of the animals were positive for *E. coli* O157:H7. The researchers then combined this incidence information with results from a study by Zhao et al. (*76*) where the distribution of *E. coli* O157:H7 concentration was determined for infected cattle. The prevalence and concentration functions were used as inputs in a processing and grinding module in the overall model. In another study, Ebel et al. (*21*) determined the prevalence of *E. coli* O157:H7 in U.S. livestock herds as a part of a larger risk assessment study for ground beef. They reported that the percentage of herds in which at least one infected animal was detected ranged from 63% in culled breeding stock herds to 88% in feedlot cattle herds. The authors also cited studies that showed a seasonal change in prevalence of 200 to > 400% increase in the number of infected animals during the summer months compared to winter months. Both herd type and seasonal prevalence were differentiated and described by probability functions in the production module of their model. Jordan et al. (*39*) did a similar herd prevalence study and used a Monte Carlo simulation to gauge incidence of *E. coli* O157:H7 in cattle. The authors defined a triangular distribution function that estimated a minimum of 45%, a mode of 63% and a maximum of 100% herds contained at least one infected animal. A more recent study at a Nebraska feedlot (*42*) found Shigatoxin-producing *E. coli* (STEC) in more than 90% of 139 cattle tested. The samples included feces, cattle oral cavity, and five locations on the hide. The results of this study demonstrated that determination of the incidence of *E. coli* O157:H7 on feces alone could underestimate the percentage of cattle infected.

Therefore, in determining incidence, factors such as sampling location on the animal, time of year, and type of analyses used may all impact inputs.

Feeding regimens may impact the presence of *E. coli* O157:H7 in beef cattle. Hovde et al. (*33*) tested the acid resistance and shedding duration of *E. coli* O157:H7 in dairy cattle given diets of hay or grain. Acid resistance of the microorganism was unaffected by diet and the authors concluded more work would be necessary before diet changes could be advocated as an intervention. Diez-Gonzalez et al. (*17*) isolated and enumerated the naturally present coliforms from digesta removed from the rectum of cattle fed diets of hay, grass, and varying amounts of rolled corn. Colonic pH of cattle fed hay or grass was greater than 7.0 and *E. coli* counts ranged from 4 to 6 log cells/g. In contrast to Hovde et al. (*33*), these authors concluded that animal diets high in grains resulted in more acid resistance of naturally present *E. coli*. Further, this could be directly related to acid resistance of pathogens and increased risk of foodborne illness for people consuming beef products. Franz et al. (*25*) compared the survival of *E. coli* O157:H7 in manure collected from cattle fed diets of straw, grass, or corn silage. Survival data for the pathogen showed the fastest decline in manure from cattle fed straw and the slowest decline for corn silage diets. The pathogen persisted in manure from corn silage (lowest pH) for up to 133 d (at 5-6 log CFU/g), compared to 84 d (at <1 log CFU/g) for straw-fed samples. As with Diez-Gonzalez et al. (*17*), these authors concluded the survival differences (based on diet) were significant in their study and that feeding hay may be a way to reduce shedding of acid-resistant *E. coli* strains. Despite the potential influence of feed on shedding of *E. coli* O157:H7 by cattle, there

are few, if any, models to predict the influence of feeding on the presence of the microorganism. With the obvious contradiction in results between the studies mentioned, any attempt at modeling such effects should be carefully validated under true system conditions.

Methods for controlling *E. coli* O157:H7 in cattle prior to slaughter may influence the incidence and concentration of this microorganism that may be present in meat products (*39, 75*). Control and/or reduction methods have included herd vaccinations, reducing visible feces via cleaning or sanitizing steps prior to slaughter, fasting prior to slaughter, changing the order of cattle introduced into processing based on visual inspection, and identifying and reducing cross-contamination from the farm to slaughter plant. Vaccination has been recommended as the most viable treatment for reduction in overall prevalence (*40, 75*). However, concerns over possible disturbance of commensal microflora and pathogen resistance are mentioned as current hindrances to vaccination strategies (*75*).

A study by Collis et al.(*14*) to evaluate cross-contamination at the live animal level investigated the spread of *E. coli* K12 strain in cattle at auction. There was a demonstrated correlation between recovery of the microorganism on hides of incoming cattle to subsequent recovery on cattle at various sites during the auction. An initial prevalence of 9.1% for incoming cattle increased to 39.4 and 54.5% in the pre-sale and post-sale pen areas (*14*). Jordan et al. (*40*) outlined a model which considered many variables for characterizing infection and possible cross-contamination of incoming cattle.

XII. Slaughter and Fabrication/Boning

There is significant impact of slaughter operations on the incidence of *E. coli* O157:H7 in beef. Potential sources of the microorganism are animal hides, gastrointestinal tract contents, and feces. Cross-contamination may occur prior to slaughter and during the slaughter process. Studies indicate a significant correlation between incoming cattle infected with *E. coli* O157:H7 and contamination of meat products at points during the slaughter and fabrication processes (*7, 22, 23, 64*). A study combining data from four Midwestern United States meat processing plants measured the frequency of *E. coli* O157:H7 and O157:non-motile strains in feces, on hides, and during processing for cattle from the same lot (*22*). The prevalence of *E. coli* was 72% in feces and 38% on hides. A similar study showed different results for fecal and hide recovery of *E. coli* O157:H7 with an overall incidence of 5.9 and 60.6%, respectively (*7*). Percentage of samples positive for the microorganism were dependent on season and ranged from 0.3 to 12.9% for feces and 29.4 to 73.5% for hides from winter to summer months, respectively. This type of information would be useful for applying risk models that take into consideration seasonal prevalence for animal infection. It would be important for the researcher to critically compare recovery methodology and the experimental design of these types of studies before combining the data.

A study conducted at an Irish beef slaughter plant tested the prevalence and level of *E. coli* O157 isolates from carcasses, beef trimmings, and boned head meat (*11*). Overall, the organism was found in 3.0% of carcass samples at levels from <0.70 to 1.41 log CFU/ g, 2.4% of beef trimmings at levels from <0.70 to 1.61 log CFU/ g, and 3.0% of

head meat samples at levels from <0.70 to 1.0 log CFU/ g. The number of trimmings tested was significant (n=1351) and they were destined for use in various minced beef products. The results of these studies indicate the importance of inclusion of sampling sites and seasonal effects when using data for risk assessment modeling. The model by Ebel et al. (*21*) addresses many of the critical control points covered in the literature both prior to and during slaughter.

Gill (*27*) evaluated the correlation between visible hide contamination and the microbiological condition of meat from cattle, sheep, and pigs and, in contrast to other studies, concluded that cleanliness of the animal had little effect on the microbiological status of the carcass. The author found relatively few studies that linked visible and microbiological contamination. Gill (*27*) warned of the relative ineffectiveness and possible redistribution of microbial load of some water washing techniques for live animals. He suggested that the skill level and care used during skinning operations were the most important factors in preventing cross-contamination between the hide surface and carcass. Though the visible contamination of incoming animals was addressed in a model by Jordan et al. (*40*), it was not cited as having a significant impact on the transmission of *E. coli* O157:H7 into the slaughter process.

Intervention methods during slaughter and carcass handling may reduce the contamination level by *E. coli* O157:H7 (*3, 4, 8, 27*). Interventions generally include removal of visibly contaminated portions of the carcass, and washing and/or chemical sanitizing, and heat treatments for animals or carcasses. A comparison of 30 lots of carcasses tested for *E. coli* during the slaughter process showed that 87% of pre-

evisceration samples were contaminated and 57% of post-evisceration (following hide removal but prior to any antimicrobial treatment) samples were contaminated (*22*). However, following antimicrobial treatments and chilling only 17% of the carcasses remained contaminated. In a similar study, carcass sampling prior to a pre-evisceration wash demonstrated an average incidence of 26.7% *E. coli* O157:H7 (*7*). Detection of *E. coli* O157:H7 on carcasses following antimicrobial intervention was similar to that found by Elder et al. (*22*) at 16.2% and only 4% of samples contained >3 CFU per 100 cm^2. Arthur et al. (*3*) tested the effectiveness of a series of antimicrobial intervention steps at 2 commercial processing plants on the incidence and concentration of *Enterobacteriaceae* and *E. coli* O157. The most probable number (MPN) indicated that 56% of positive incoming hides were contaminated at levels below the MPN detection limit, 41% between 60 and 9,999 MPN/100 cm^2, and 3% at >10,000 MPN/100 cm^2. Following interventions, the post-evisceration prevalence decreased dramatically at both facilities, to an overall average of 3.8%. While the mean incoming prevalence of *E. coli* O157 was 76%, following the intervention processes, carcasses at both facilities were negative for the presence of the pathogen after leaving the chill cooler. Interventions included high-pressure water rinsing and steam vacuuming of hides as well as a lactic acid treatment, a 90°C water spray, a peroxyacetic acid solution spray and steam pasteurization of carcasses. Thus, it is important to accurately quantify microbial reductions due to intervention methods during processing. Few models include detailed inputs that differentiate between decontamination procedures. The

model by Cassin et al. (*13*) applied a simple uniform distribution function, ranging from a 1 to 2.5 log CFU/cm^2 reduction due to decontamination treatments.

XIII. Processing of Ground Beef

If *E. coli* O157:H7 is present on a beef carcass, what are the chances that the microorganism can be transferred to a packaged meat product and what conditions influence the potential levels of that contamination? To determine this, we need to know the potential sources of contamination and cross-contamination during processing, the potential for growth of the microorganism in the product, and the influence of processing techniques and additives on microbial growth. The processing and grinding module in the model by Cassin et al. (*13*) includes one such attempt at detailing inputs which characterize the transfer of the pathogen at specific points in a hypothetical beef processing plant.

Hussein and Bollinger (*34*) reviewed the prevalence of both O157 and non-O157 Shiga toxin producing *E. coli* strains (STEC) in processed meats and potential sources of contamination from the slaughter operation. They found that studies showed an overall prevalence of O157 STEC strains ranging from 0.01 to 43.4% on whole carcasses, 0.1 to 54.2% in ground beef, and 1.1 to 36.0% on unspecified retail cuts. Non-O157 STEC prevalence was slightly lower in ground beef (2.4 to 30.0%), but significantly higher from other sources such as sausage (17.0 to 49.2%), whole carcasses (1.7 to 58.0%), and on unspecified retail cuts (11.4 to 49.6%). The primary concern cited for ground beef was

the mixing of contaminated fecal material from the surface of meat during the grinding process.

Stopforth et al. (*65*) studied the potential for cross-contamination of meat by *E. coli* O157:H7 through contamination of food contact surfaces of processing equipment. They inoculated the runoff fluid from a meat decontamination spray process with acid-adapted *E. coli* O157:H7 to evaluate survival and biofilm formation on stainless steel surfaces. The researchers combined spray runoff fluids with water, 2% acetic, and 2% lactic acid solutions in 3 dilutions, (1/9, 1/49, and 1/99 vol acid/vol runoff). The tests were conducted at 15°C for up to 14 d. Results for acid solutions showed detection levels >1.3 log CFU/ cm^2 for up to 4 d in 1/9 dilutions and detection from 2.5 to 4.3 log CFU/ cm^2 up to 14 d in the higher dilutions (1/49, 1/99). Water washes, with no acid stress, resulted in biofilms that were 2 logs higher in concentration, over a 2- to 7-day period. After 14 d the competitive growth of *Pseudomonas* spp. was shown to be predominant in the biofilms under all conditions. Since many models make or imply the assumption of adequate cleaning and sanitizing practices between production runs, a major concern is the ability of the pathogen to form such biofilms under processing conditions. If cleaning and sanitizing are not adequate, model input and transfer values for simulation runs become skewed and less accurate.

Microbial growth on meat can be extensive given the high water activity of muscle tissue (0.99) and the ready availability of glycogen, various peptides, and free amino acids (*18*). However, meat processors sometimes incorporate “hurdles” or interventions into their process to curb the growth of or eliminate both spoilage and

pathogenic bacteria. The concept of "hurdle technology" has been well defined (*44, 45*). The one drawback to this technology is that it is difficult to quantify and is therefore generally used qualitatively. However, McMeekin et al. (*51*) have discussed potential quantification of the technique through modeling of the growth/no-growth interface. A number of studies have modeled microbial growth response to multiple hurdles such as those used for meat, for example, temperature, pH, and antimicrobials (*48, 51, 58*). Using a square root model, combined with logistic regression techniques, Presser et al. (*58*) developed a growth/no-growth model for a strain of *E. coli* (M23). The model considered the water activity, temperature, pH, and effects of undissociated lactic acid concentration on growth of the microorganism. The latter was determined by comparing solution pH to the pK_a of lactic acid. The right-hand-side of the equation takes the form of a square root numerical transformation of these effects and the left-hand-side is a binomial distribution (logistic regression) or odds of the outcome. The growth interface was shown to be independent of temperature between 15°C and 37°C. Growth at 10°C was observed at pH 4 to 5.4 for lactic acid ranging from 0 to 200 mM, respectively. These pHs are below the likely surface pH of raw meat following rigor and glycolysis (pH 5.5 to 5.8) (*18*). McKellar and Lu (*48*) utilized a polynomial equation in a logistic regression model to predict the probability of growth of *E. coli* O157:H7 as a function of temperature, pH, salt, sucrose, and acetic acid concentration. Growth inhibition in this study was observed at 10°C, between pH 4 and 5 for a 0.5% concentration of salt and 0.5% acetic acid. The application of such detailed growth models into larger risk assessment simulations can be complicated. In a quantitative risk

assessment model by Duffy and Schaffner (*20*), the survival and growth of *E. coli* O157:H7 in refrigerated apple cider was modeled as a histogram of data collected from various studies. In contrast, Cassin et al. (*13*) combined multiple inputs to determine the coefficients for a Gompertz equation and calculate growth during retail storage and display. In the case of minimal processing and stress adaptation, the development of predictive models that consider the pH and/or temperature-time history of the pathogen prior to inactivation processes could be beneficial.

XIV. Transportation, Retail, and Domestic Storage

Whether final ground beef packaging occurs at the processing plant or the point of sale, the major concerns after grinding are cross-contamination and temperature abuse. Of these, temperature abuse would likely result in greater microbial concentration at the point of preparation and consumption. A study by Rajkowski and Marmer (*59*) observed the growth response of *E. coli* O157:H7 under variable temperature abuse scenarios. The study was conducted in microbiological broth at various pHs (5-7) and NaCl concentrations (0.5-3%). Five temperature fluctuation regimes, each lasting for 6 h, were conducted with ranges of 4 to 12, 4 to 19, 4 to 28, 8 to 19, and 12 to 28°C. Growth was observed up to 21 d under all conditions tested. One concern was the observation of growth at 8°C given sufficient lag time. The general findings of the study indicated that growth curves more closely approximated expected results of the high end of each temperature fluctuation regime, rather than the average.

This would be significant when applying growth models to measured temperature fluctuations. Integration of the temperature fluctuation profile would give equal significance to all temperature points (in the range) in order to calculate growth parameters.

Proper temperature control between slaughter and commercial retail is critical in controlling bacterial growth. Researchers have studied temperature fluctuation of beef products during transport between processing facilities and through retail delivery (*28, 29*). Gill et al. (*26*) collected surface and central temperatures of bulk containers (450 kg) of large beef cuts and carcasses destined for further processing to evaluate the likelihood of *E. coli* growth. Temperatures were taken when containers were filled at packing plants and upon arrival at 2 further processing facilities. Duration times from the point of loading containers to unloading for further processing ranged from 20 to 120 h. Some variability in temperatures was observed in 2 of 5 packing plants, although all surface and central temperatures averaged < 5^{o}C upon arrival to processing facilities. The authors concluded there was no significant growth potential for *E. coli* during storage and transport between facilities in this study. They went on to conclude that packing temperatures of >7^{o}C, under slow cooling conditions, would constitute concern for growth.

Surveys of retail storage and display cases provide valuable inputs for risk assessment models. As previously mentioned, growth of *E. coli* O157:H7 in retail display cabinets has been modeled and reported (*12*). Several studies have analyzed temperature fluctuations and time on display for beef products during retail display (*30-*

32). Greer et al. (*32*) used temperature logging devices to study fluctuation in conditions at various locations inside a commercial display cabinet. Meat surface, deep muscle, and air temperatures were logged in the front, center, and rear of the cabinet at heights of 17 to 13 cm below and 4 cm above the recommended fill line. The highest average meat surface temperatures were above the fill line in the center position (6.1-10^{o}C) and there was little difference between surface and deep muscle temperature values. The average meat surface temperature across all conditions was approximately 6^{o}C and the authors noted good correlation between surface temperatures from 4 to 8^{o}C and the growth of *E. coli*. The authors developed a predictive regression model which showed approximately 2 generations of *E. coli* growth in 48 h at a surface temperature of 6^{o}C. It was concluded that meat surface temperatures below 4^{o}C would be necessary to minimize *E. coli* growth. Although this study was conducted in a single retail display cabinet, the researchers surveyed 4 additional cabinets for incoming air and rack level average temperatures and observed ranges of -3.3 to 1.8^{o}C and -0.6 to 7.5^{o}C, respectively. The authors noted higher temperature fluctuations during the automatic defrost cycle of the cabinets, which occurred once every 12 h. Given these temperature ranges, it is possible that growth can occur at the retail level and this information must be addressed in risk assessment modeling.

A likely source of temperature abuse that would be difficult to quantify or control is that which takes place in the home. A recent study by Kosa et al. (*43*) sampled 200 household refrigerators in Tennessee, Kansas, and Florida. Temperature fluctuations and internal temperature response for various foods were compared.

Greater than 12% of refrigerators tested had mean temperatures above 7.3^{o}C on the bottom shelf of the door panel, while all refrigerators showed mean values below 7.3^{o}C on the top shelf inside the cabinet. The researchers found that the recommended mean temperature of 4.4^{o}C was achieved 91% of the time on top shelves, 79% of the time on bottom shelves, and 45% of the time on the bottom shelf in the refrigerator door. Mean temperatures recorded by thermocouples placed in commercially packaged hot dogs and yogurt cups indicated values greater than 7.3^{o}C only 3% and 1% of the time, respectively. A key recommendation by the researchers, aside from consumer awareness of adequate chilled storage, was the need to place a thermometer in the door of the refrigerator and monitor it regularly. A study conducted in Greece which surveyed temperature distributions of 250 household refrigerators found that nearly 10% had average temperatures above 10°C (*63*). Using Monte Carlo simulations, the researchers compared the growth rates of normal spoilage bacteria to that of *Listeria monocytogenes* and concluded it would take large initial pathogen contamination to result in unacceptable levels. Risk will ultimately depend on the food item and preparation techniques, but such comparisons of pathogen to spoilage microflora could offer valuable information to risk assessment regimes.

XV. Consumer Preparation

Two major risk factors for exposure to *E. coli* O157:H7 at the consumer preparation level are cross-contamination and lack of adequate cooking. There is

relatively little quantitative information on cross-contamination via food to food or surface to food of *E. coli* O157:H7. Therefore, this is a research need for the development of adequate risk models. In contrast, there are considerable data on the influence of cooking temperatures on inactivation of *E. coli* O157:H7. Ground beef is generally regarded as safe after proper cooking temperatures are reached throughout the product. Ground beef risk assessment studies (*13, 21*) have included modules which calculate the reduction of *E. coli* O157:H7 using input probability distributions of likely cooking temperatures achieved during preparation. Cassin et al. (*13*) combined 2 studies, one citing the percentage of preferred level of degree of doneness by the consuming population (3% rare, 16.1% medium rare, 17.9% medium, 23.4% medium well, and 39.6% well done) and another which attached a relative temperature level to these preferences (54.4, 58.6, 62.7, 65.6, and 68.3°C, respectively). Combining this information into a custom probability histogram and using the temperature values to calculate the level of inactivation with the PMP (*70*), the researchers predicted the amount of the pathogen likely to survive the cooking process. The basis of the calculation in the PMP was a log-linear model by Juneja et al. (*41*), where the researchers measured *E. coli* O157:H7 survival in hamburger cooked to internal temperatures ranging between 56.1 to 74.4°C. Validation of such calculations, within the framework of large Monte Carlo simulations, is crucial in making recommendations from model results. The overall prevalence and concentration of the pathogen after cooking was combined with a probability distribution of the likely amount consumed by the target population and these numbers were entered into a dose-response module.

The overall results included the probability of illness, followed by a percentage calculation of individuals suffering further complications (HUS) and possible death. The probability of an adult becoming ill from a single hamburger meal was estimated to be 5.1×10^{-5} compared to 3.7×10^{-5} for a child. The subsequent probabilities for developing HUS and mortality for children were calculated to be 3.7×10^{-6} and 1.9×10^{-7}, respectively. Dose-response and risk estimates were also included in the model by Ebel et al. (*21*). Duffy et al. (*19*) summarized hypothetical risk mitigation strategies, taken from 2 risk models, which could reduce the potential for illness from pathogenic *E. coli* on ground beef.

XVI. Summary

The importance of using modeling techniques for safety and risk prediction in the food supply is strongly supported from the incidence of foodborne outbreaks worldwide. An overarching risk assessment model which addresses incidence and concentration of foodborne pathogens, microbial growth, microbial inactivation, cross contamination and dose response of the host from production to the consumer should be the ultimate goal. Additionally, while pathogenic and spoilage bacteria have been emphasized in predictive microbiology, there are opportunities to address the impact from other potentially hazardous contaminating agents, such as viruses, parasites, and chemical compounds. It is also important that those researchers who choose not to

model their results are aware of the significance and format of their findings in order to facilitate the use of their data by others.

The choice and combination of modeling techniques (regression models and/or Monte Carlo simulations) will determine the form and amount of useable research available. Caution should be taken in the grouping of available research, keeping in mind similar techniques and comparable process conditions. When developing tertiary models with underlying code and mathematical techniques, it is advisable to offer key choices where specific actions dictate results, such as the choice of specific antimicrobial interventions. The use of general probability distribution functions for inputs where there is little research available to validate the results will likely add uncertainty. The researcher must continually question the use of a model and keep in mind the availability of data to validate any conclusions.

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Appendix

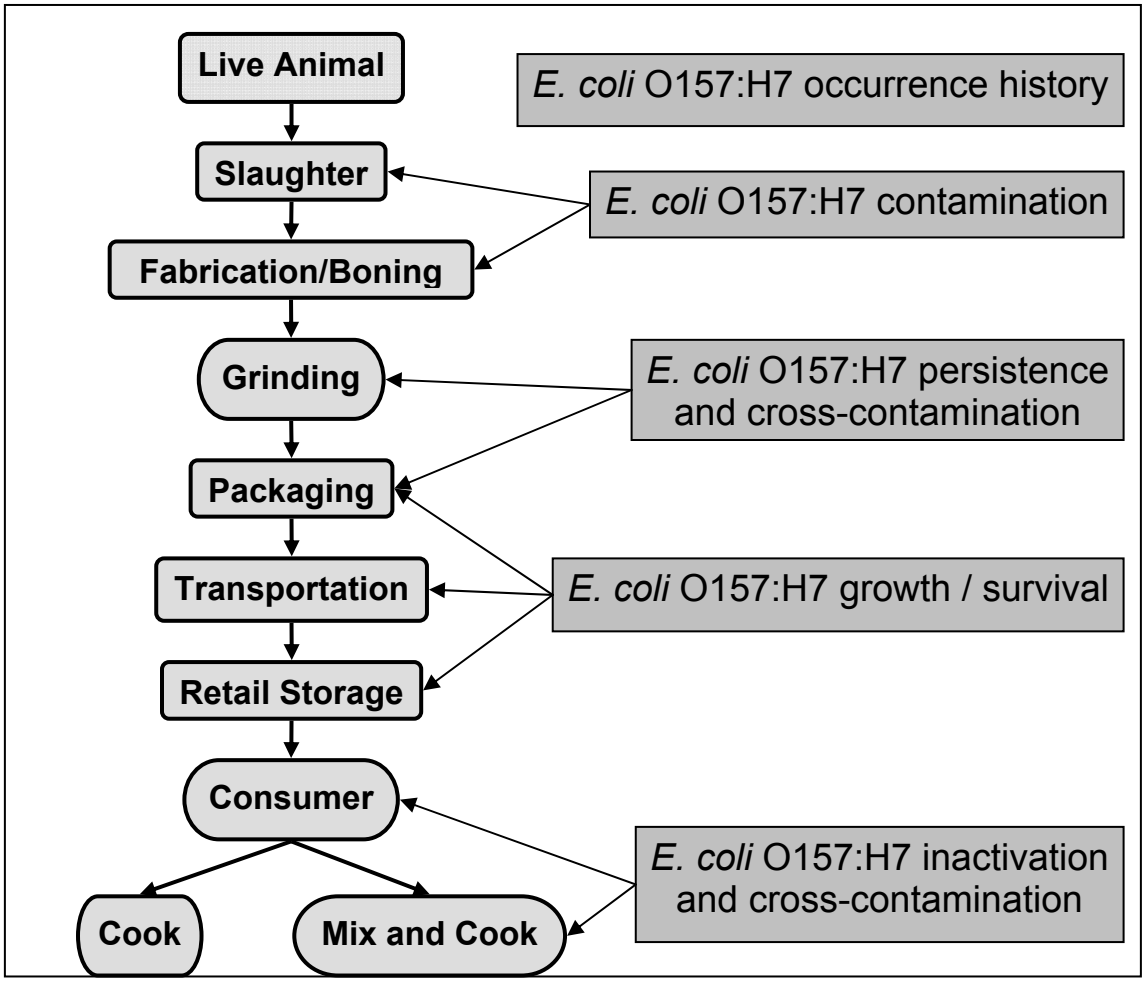

Figure 1 Potential Escherichia coli O157:H7 inputs in the ground beef production, process, and consumer handling continuum from "farm to table"

PART TWO: *ESCHERICHIA COLI* THERMAL INACTIVATION RELATIVE TO PHYSIOLOGICAL STATE

Abstract

Studies have explored the use of various non-linear regression techniques to better describe shoulder and/or tailing effects in survivor curves. Researchers have compiled and developed a number of diverse models for describing microbial inactivation and presented 'goodness of fit' analysis to compare them. However, varying physiological states of microorganisms could affect the measured response in a particular population and add uncertainty to results from predictive models. The objective of this study was to determine if the shape and magnitude of the survivor curve is possibly the result of the physiological state, relative to growth conditions, of microbial cells at the time of heat exposure. Inactivation tests were performed using *Escherichia coli* strain K12 in triplicate for three growth conditions: traditionally or statically grown cells, chemostat-grown cells, and chemostat-grown cells with buffered (pH 6.5) feed media to match the pH of the statically grown cells. Chemostat cells were significantly less heat resistant than the traditional or buffered chemostat cells at 58°C. Regression analysis was performed using the GInaFiT freeware tool for Microsoft® Excel. A non-linear Weibull model, capable of fitting tailing effects, was effective for describing both the traditional and buffered chemostat cells. The log-linear response best described inactivation of the non-buffered chemostat cells. Results showed differences in the inactivation response of microbial cells depending on their physiological state. The use of proper regression tools can result in more accurate characterization of the response of a given organism under specified conditions. However, a key consideration

for the use of any model should include a comprehensive understanding of the growth and inactivation conditions used to generate thermal inactivation data.

I. Introduction

The log-linear approach to analyzing inactivation of microorganisms has long been the primary modeling tool for thermal process validation in the food industry. Recent studies have explored the use of various non-log-linear regression techniques to better describe inactivation data which are curvilinear or show shoulder and/or tailing effects in survivor curves. Researchers have compiled and presented a number of diverse models for describing microbial inactivation and presented 'goodness of fit' analyses to compare them (*4-6, 17*).

Non-log-linear modeling techniques, such as those using the Weibull function (*8-10, 14*), offer flexibility over the first order model due to the use of both a rate constant and a shape factor which characterize population density versus time. The shape factor gives the function the ability to fit both concave and convex shapes. Fixing the Weibull shape factor to a value of 1.0 makes the Weibull function equivalent to the first order model, with the appropriate mathematical techniques.

A number of researchers have discussed the 'non-log-linear' effect seen in thermal death curves for both the inactivation of vegetative cells and spores (*3-5, 12, 15*). Possible causes for non-linearity include, mixtures of strains which vary in heat resistance (genetic heterogeneity), 'lethal hit' theory, adaptation during growth and/or stress (temperature, pH, water activity), clumping of cells/spores during processing, accessibility to heat (food component effects), and recovery methods for enumeration (selective vs. non-selective media).

Varying physiological responses of microorganisms could affect the measured response in a particular population and add uncertainty to results from predictive models. The objective of this study was to determine if the shape and magnitude of the survivor curve is possibly the result of the physiological state, relative to growth conditions, of microbial cells at the time of heat exposure.

II. Materials and Methods

A. Inoculum. *E. coli* (strain K12, ATCC 10798) was grown aerobically and statically (traditional method) in tryptic soy broth (TSB, Difco, Sparks, MD) for 24 h at 35°C for two consecutive transfers prior to use. This culture was also used to inoculate a chemostat vessel.

B. Chemostat method. The chemostat enabled a control framework to achieve a quasi-constant growth rate of bacteria and nutrient concentration, allowing bacteria to stay in a sustained growth phase. The initial nutrient concentration was supplied by TSB at 27.5 g/l of deionized water. A model C30/32 BioFlo chemostat was used (New Brunswick Scientific, Edison, NJ), having a reaction chamber capable of producing 350ml of cell culture. Product feed was maintained with a MasterFlex Peristaltic variable speed pump unit, utilizing a model 7014-20 pump head (Cole-Parmer Instrument Co., Chicago, IL). Approximately 3 - 4 h following inoculation with ca. 1 ml of culture solution into ca. 200 ml of nutrient (in growth chamber), significant turbidity was observed and the nutrient pump started (at a rate of 0.6 ml/min). Agitation in the reaction vessel was

maintained at 400 rpm and filtered air introduced at ca. 0.1 liter/min. The reaction chamber was maintained at 34°C using a controlled water jacket heated by a water bath adjacent to the unit. Test culture was extracted through a sample port which collected culture from the middle of the growth chamber.

C. Chemostat method (buffered). Growth conditions were maintained as described above with the addition of a phosphate buffer system to maintain the feed media pH at 6.5. The TSB media was buffered to pH 6.5 using 0.1M phosphate buffer.

D. Inactivation procedure. Inactivation tests were performed in triplicate for each growth condition (traditionally grown cells, chemostat cells, and chemostat cells with buffered feed media). Culture from the preparation methods was diluted 1:10 with 0.1% peptone (Sigma, St. Louis, MO) and placed into closed screw-capped glass vials (12 x 35 mm). Initial population control counts were made on this dilution. The total volume added to each vial was 2.1 ml. In order to minimize individual vial preparation error, the 1:10 dilution of cell culture was achieved by diluting cells into a large enough volume of peptone buffer to fill all test vials. The initial diluent pH was ca. 7 for both cell types. An open bath circulator (Haake model V26, Karlsruhe, Germany) was used to immerse and maintain two perforated aluminum baskets containing the test vials at a constant temperature (58°C) prior to extraction at pre-set time intervals. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA). Come-up times for the vials were determined by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the center of 2 vials filled with peptone and submerged into the water bath. The thermocouples were connected

to an Omega OMB-Chartscan-1400 portable data recorder utilizing ChartView Plus software (Omega Engineering, Inc., Stamford, CT). Vials of each culture preparation were extracted from the bath at prescribed time points. The initial time point was 1 min followed by 3 min and 3 min intervals for a total time of 36 min. Upon removal, sample vials were immediately cooled in an ice water bath to stop further thermal inactivation.

E. Enumeration of survivors. After the vials were cooled, samples were taken within 30 - 40 min. The earlier time points were serially diluted as needed to achieve readable plates for enumeration. Each replication was plated on tryptic soy agar (TSA, Difco) in duplicate using a spiral plater (Don Whitley Scientific Limited, Yorkshire, UK). Plates were incubated for 24 h at 35°C before enumeration of *E. coli* using a Protocol automatic plate counter (Synoptics Ltd., Cambridge, UK). For later time points, where specific log population was below log 3 CFU/ml, undiluted samples were spread plated (0.2 to 0.4 ml) on TSA plates for more precise counts.

F. Curve fitting. Regression analysis was performed using the GInaFiT freeware tool for Microsoft® Excel (*6*), as well as the standard curve fitting capabilities of Excel. Geeraerd et al. (*6*) created a freeware Add-in for Microsoft® Excel (GInaFiT) which bundled a group of static models capable of fitting 8 common shapes found in microbial inactivation data. The shapes include log-linear, log-linear with a tail and/or shoulder, the Weibull function with and without a fixed shape factor and tailing, and bi-phasic applications.

The user enters experimental data relating time and $\log_{10}$ population counts and is given the choice of both linear and non-linear approaches, with and without tail

effects. The authors give the source and describe the basic governing equations for the models and the output includes parameter estimates with standard error, overall model Sum of Squared Errors (SSE), Mean Sum of Squared Errors (MSE), and Root Mean Sum of Squared Errors (RMSE). Two of the nine models describing microbial survival in the GInaFiT tool were used in the analysis of this data: 1) the model presented by Bigelow and Esty in 1920 for log-linear response (which has the functional form):

$$\log_{10}(N(t)) = \log_{10}(N(0)) - \frac{t}{D}$$

where *N(t)* is the observed population at time *t* of the process and *N(0)* is the initial population (*D* -value is the time for a 90% population reduction at the isothermal process temperature), and 2) the Albert and Mafart model (2003) which uses the non-linear Weibull function with the addition of a tailing effect (which has the functional form):

$$\log_{10}(N(t)) = \log_{10}\left[(N(0) - Nres)\cdot 10^{-\left(\frac{t}{\delta}\right)^{p}} + Nres\right]$$

where *Nres* represents a population tail value, *δ* is the hazard rate parameter and *p* is the shape factor.

The Weibull distribution can take the form as described by Mafart et al. (*11*), where the hazard rate parameter (*δ*) is referred to as the 'time of first decimal reduction'. Fixing the Weibull shape factor (*p*) to a value of 1.0 makes the hazard rate function mathematically equivalent to the D-value. In addition to the fit statistics provided by the freeware tool, a 'goodness of fit' tool recommended by several researchers (*2, 3, 10*)

was used. It is a mathematical technique that relates the percentage of variance (%V) accounted for by the model (based on number of terms):

$$\%V = \left[1 - \frac{(1 - r^2)(n-1)}{n - N_T - 1}\right] \times 100$$

Where *r* is the correlation coefficient, *n* is the number of data points and N_T is the number of model equation terms. This coefficient takes into account the complexity of the model and the population of data used to describe it. As the number of observations *n* increases, the number of terms (N_T) has less of an effect on the 'goodness of fit'.

III. Results and Discussion

Traditionally grown cells, under static conditions in an incubator, likely followed a more anaerobic, fermentative pathway which was evidenced by a lowering of the pH of the growth media from ca. 7.2 to 6.2. When added to 0.1% peptone for heating, the pH recovered to ca. 6.5 for traditional cells. The non-buffered chemostat cells, which were exposed to constant mixing and introduction of air, likely followed an aerobic pathway and the pH of the growth media actually increased to ca. 8.3 (approximate pH in peptone diluent for heating of 7.6 to 7.7). For phosphate-buffered chemostat cells, the final pH in diluent remained ca. 6.5 (equivalent to traditional cells during inactivation).

Figure 2 shows the scatter plot and regression line (GInaFiT tool) for heat inactivation experiments performed on traditionally grown cells at 58°C. It should be

noted that the vials containing the cells reached process temperature in 60 to 65 sec at 58°C. The scatter plot for this situation shows significant variability, with a distinctive non-linear response. The Albert and Mafart model (2003) was used to fit the data, resulting in the highest correlation (R^2 = 0.94). The magnitude of response (utilizing the D-value method) was also higher than that of the unbuffered chemostat cells (Fig. 3), with a corresponding $D_{58°C}$ of 4.46 min, measured as 2.303/kmax when using the log-linear approach to approximate the entire curve. The kmax value (maximum rate of inactivation) and regression fit data are given by the GInaFiT tool. Traditionally grown cells showed a significant tailing effect at 27 to 33 min of exposure.

Figure 3 shows the scatter plot and regression line for heat inactivation experiments performed on non-buffered chemostat cells at 58°C. The log-linear response best describes the inactivation of these cells under the prescribed heating conditions. The corresponding $D_{58°C}$ was 2.22 min. The scatter plot shows strong correlation (R^2 = 0.97) and the resulting regression parameters would be ideal for predictive modeling in similar experiments. In one of the chemostat runs, there were no countable colonies beyond the 9 min, which does result in fewer data points for the later times.

When the chemostat was buffered to ca. pH 6.5, the response was similar to that of the traditionally grown cells (Fig. 4). A notable difference is a slight decrease in variability seen in the scatter of data points. Due to repeatability for cells grown under these conditions (as well as non-buffered chemostat cells), many of the population values overlap and give the appearance of fewer data points per time point. The Albert

and Mafart model (*1*) was also used to fit the buffered chemostat cells, resulting in slightly higher correlation than traditional cells (R^2 = 0.97 vs. 0.94, respectively) and lower response magnitude ($D_{58°C}$ of 3.41 vs. 4.46 min, respectively).

The D-value (log-linear) approach would not be recommended for the analysis of either the traditionally grown or buffered chemostat cells in this study. The respective values are given here as a means of comparison (magnitude response). It should be noted the log-linear regression of buffered chemostat cells resulted in non-conservative estimation (predicted counts lower than observed counts) of population counts at the later time points (Fig. 5). This is possibly the effect of more pronounced tailing for these cells, resulting in a stronger skew when fitting the data with a linear model.

Table 1 shows the fit statistics for the chosen models for each cell growth method. The Root Mean Sum of Squared Errors (RMSSE) are slightly lower for chemostat cells, represented in Figures 3 and 4, as compared with traditional cells. The R-Squared values in all cases roughly mirror the variance percentage accounted for (%V) by the respective models. The %V statistic is more applicable in situations where there is limited data and/or where a model has many terms. For sensitivity comparison, the buffered chemostat cells were also fit with the log-linear model. The resulting RMSSE was higher than that of the non-linear model, 0.61 versus 0.459 for the linear versus Weibull model. The R-Squared and %V values also dropped two percentage points, indicating a less accurate approximation of the curve. While these values do not represent large deviations from the optimal fit statistics, the risk of underestimating the true response is more significant (Figure 5). As discussed earlier, the use of the log-linear

approach in this case could result in possible under-processing for the surviving cells represented by the tailing effect at the 30 and 33 minute time points.

The similarity in response of traditionally grown cells to buffered chemostat cells has been cited in previous studies. Humpheson et al. (*8*) utilized a chemostat to test inactivation of *Salmonella* Enteritidis PT4 relative to statically grown cells and found no significant difference in response when buffering the chemostat feed media to pH 6.5 (±0.25). The researchers introduced prepared cell cultures into preheated inactivation media and observed similar tailing effects and response magnitude for both growth types.

Another study by Kaur et al. (*9*) showed a significant difference in heat resistance for cell inactivation relative to growth phase. *E. coli* O157:H7 (NCTC 12079) was grown at 37°C with shaking (150 rpm) and heat resistance evaluated after 3 h (log phase) or 48 h (late stationary phase). Depending on the inactivation temperature used, the authors showed cell population differences ranging from 2 to 4 log CFU/ml at some time points. While the shape of the inactivation curves for both growth phases were non-linear, the comparable magnitude of response was significant.

The non-buffered chemostat cells in the current study showed a strong log-linear response and required inactivation processes of half the time required for the other growth methods. A possible cause of the non-linear response of traditional and buffered chemostat cells could result from partial acid-adaptation at pH 6.5, both naturally occurring due to fermentation under static conditions and induced in the more controlled conditions of the chemostat. The equilibrium pH during growth and at the

time of inactivation appears to have the most significant influence on both shape and magnitude response of the inactivation curve.

Results indicate there are differences in the inactivation response of microbial cells depending on their physiological state. The difference in physiological state in this experiment results from traditionally grown cells in a static, nutrient limiting environment, compared to chemostat cells (both buffered and non-buffered) in an aerobic, unlimited nutrient environment. Additionally, the use of regression tools, such as GInaFiT (*6*) can result in more accurate characterization of the response of a given organism under specified conditions. A key consideration for the use of any model is a complete characterization of the growth and inactivation conditions leading to specific output results. It is crucial that such information is properly taken into account when modeling validation experiments to ensure adequate food safety.

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Appendix

Table 1 Fit statistics for the compared growth methods.

Growth method + Model	RMSSE	R-Square	%V
Traditional cells Weibull plus tail[1]	0.543	0.94	93.66
Chemostat cells Log-linear[2]	0.452	0.97	96.85
Chemostat cells (Buffered) Weibull plus tail[1]	0.459	0.97	96.83
Chemostat cells (Buffered) Log-linear[2]	0.61	0.95	94.89

[1] Albert and Mafart model (2003): Weibull (non-linear) plus tailing
[2] Bigelow and Esty (1920): Log-linear
RMSSE = Root Mean Sum of Squared Errors;
%V = percentage of variance accounted by in the model.

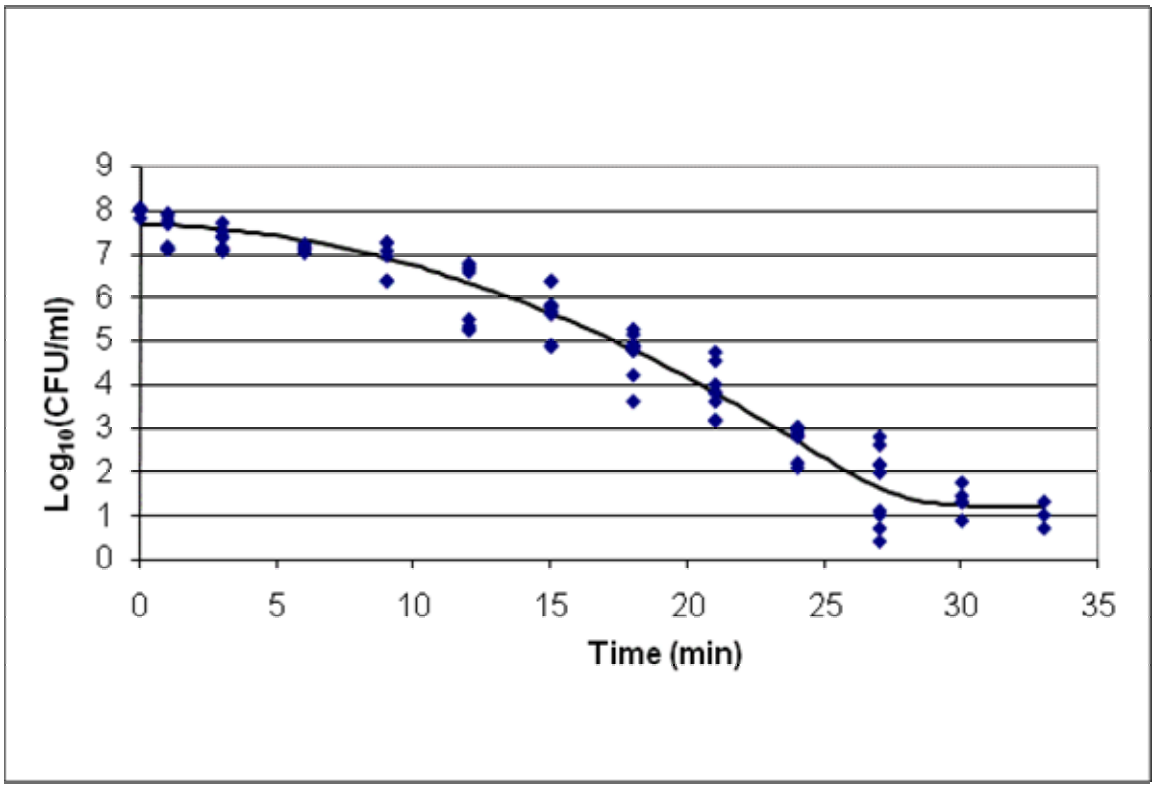

Figure 2 Inactivation of *Escherichia coli* K12 *in vitro* at 58°C, grown 24 h under static conditions, ~ pH 6.2 during growth using the Weibull plus tail model

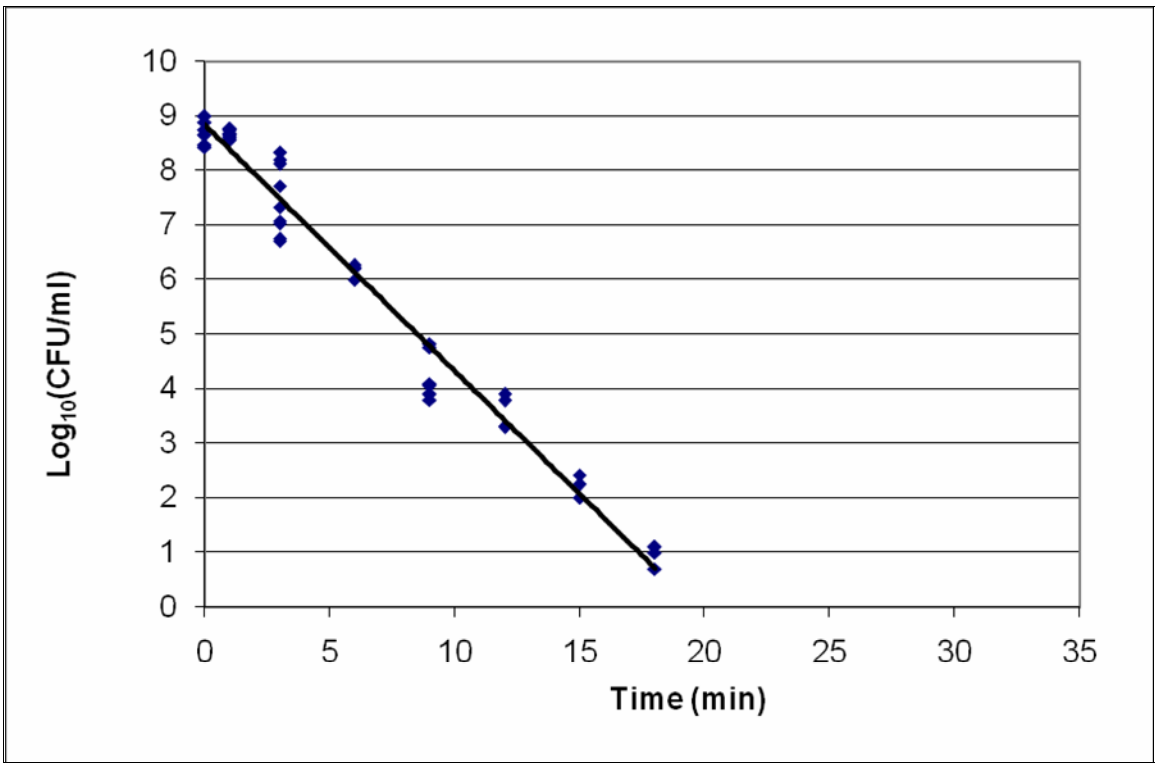

Figure 3 Inactivation of *Escherichia coli* K12 *in vitro* at 58°C, grown in a chemostat method, ~ pH 8.3 during growth using the log-linear model

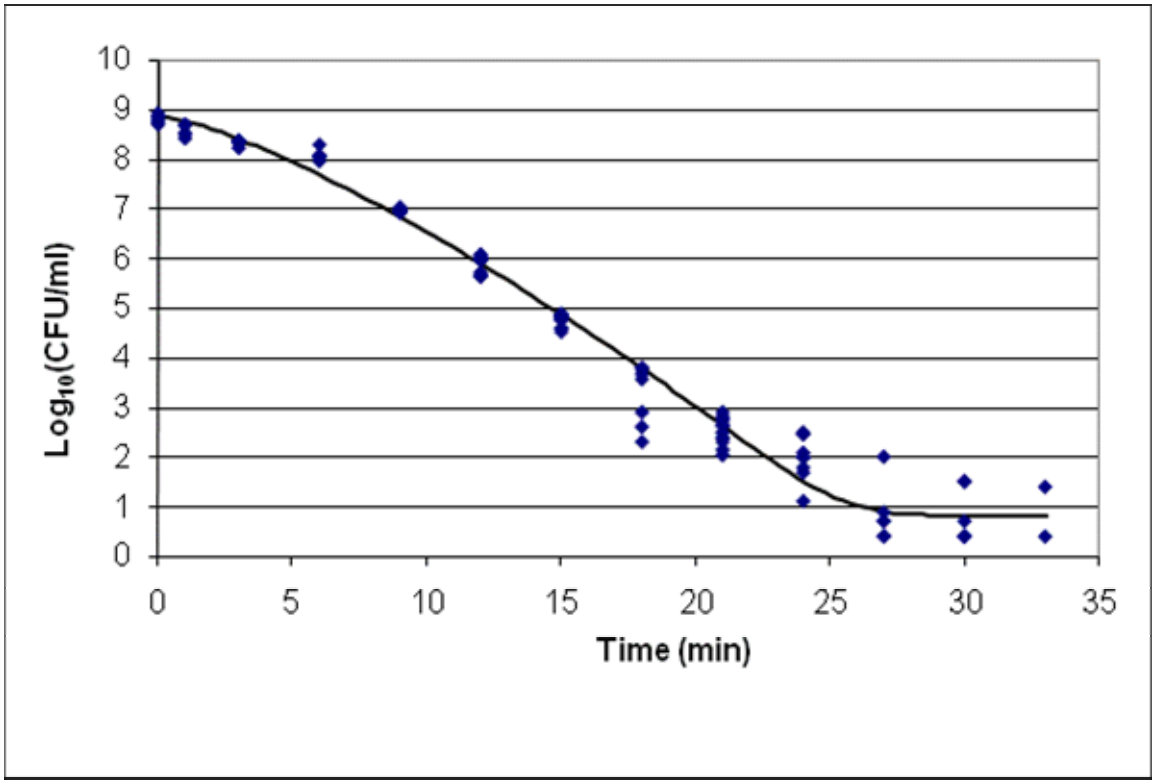

Figure 4 Inactivation of *Escherichia coli* K12 *in vitro* at 58°C grown in a chemostat with buffering to pH 6.5 using the Weibull plus tail model

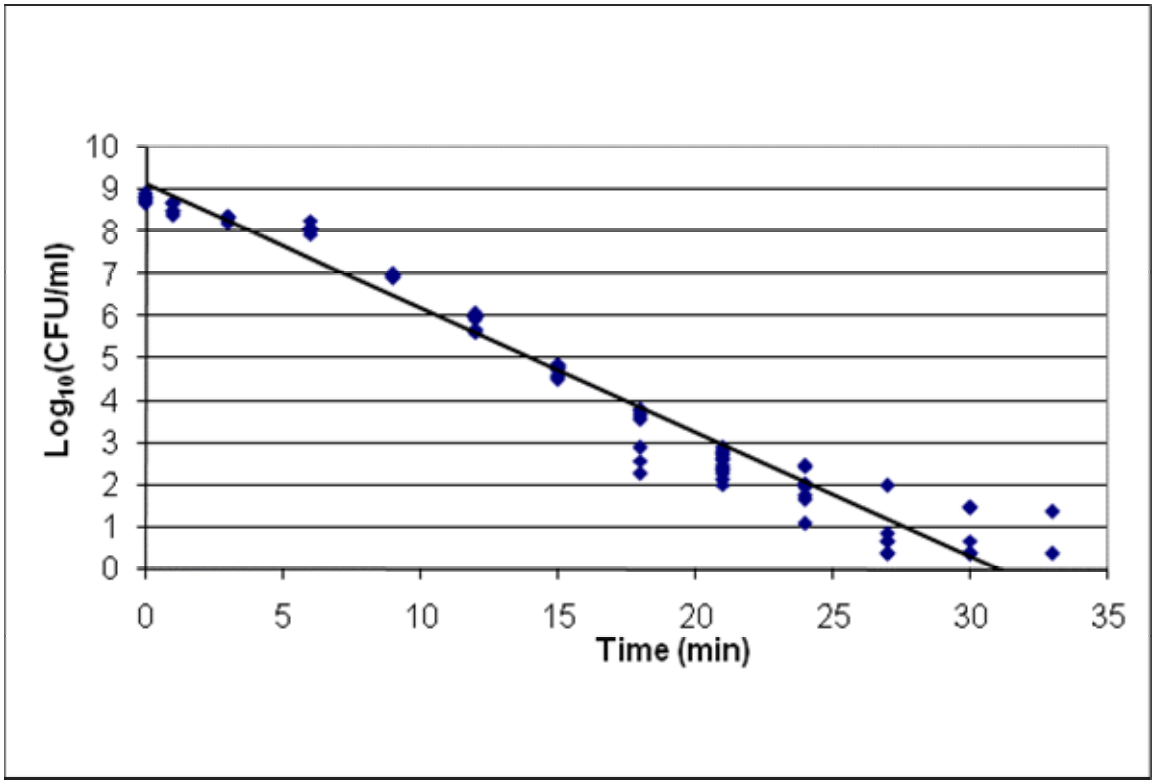

Figure 5 Inactivation of *Escherichia coli* K12 *in vitro* at 58°C grown in a chemostat with buffering to pH 6.5 using the log-linear model

PART THREE: THERMAL INACTIVATION OF *ESCHERICHIA COLI* O157:H7 WHEN GROWN STATICALLY OR CONTINUOUSLY IN A CHEMOSTAT

Abstract

The objective of this study was to determine if the survivor curves for thermally inactivated *Escherichia coli* O157:H7 were affected by the physiological state of the cells relative to growth conditions and pH of the heating menstruum. A comparison was made between the commonly used log-linear model and the non log-linear Weibull approach. Cells were grown traditionally (statically) in culture tubes or under controlled conditions in an aerobic chemostat in tryptic soy broth (pH 7.2). Heating menstruum was unbuffered 0.1% peptone or 0.1 M phosphate buffer (pH 7.0). Thermal inactivation of the cells was carried out at 58, 59, 60 and 61°C and recovery was on a non-selective tryptic soy agar. Longer inactivation times for traditionally-grown cells indicated potential stress adaptation of these cells. This was more prevalent at 58°C. Shape response was also significantly different, with traditionally-grown cells exhibiting reducing thermal resistance over time and chemostat cells showing the opposite effect. Buffering the heating menstruum to ca. pH 7 for both traditionally-grown and chemostat cells resulted in inactivation curves which showed less variability or scatter of data points. Time to specific log reduction values (t_d) for the Weibull model were either conservative relative to the log-linear model for full population reduction but less so when considering partial population reduction. The Weibull model offered the most accurate fit of the data in all cases, especially considering that the log-linear model is equivalent to the Weibull model with a fixed shape factor of 1. The determination of z-

value for the log-linear model showed a strong correlation between log D-value and process temperature.

I. Introduction

The traditional log-linear modeling of thermal inactivation is based on the assumption that heat provides irreversible first order reaction kinetics that inactivate microbial populations(*13*). The linear slope of the semi-log plot of population versus process time provides the decimal reduction constant (D-value) which characterizes the time required to reduce a microbial population by 90% under specific isothermal conditions. This has long been the accepted basis used for process validation in the food industry.

A number of researchers have proposed that the thermal inactivation of vegetative cells and spores can be described as a ”non-log-linear” effect *(3-5, 12, 15)*. An extensive discussion, describing the applicability of non-log-linear analysis for thermal inactivation, is given by van Boekel *(14)* where the author describes the probabilistic response of specific microbial populations. The author performs regression analysis, using the Weibull function, on 55 previously published data sets and provides parameter estimates for the model equation. Comparisons included specific microorganisms, various heating menstrua, and a range of process temperatures. The Weibull function is a non-linear power-law equation which allows linear, concave, and convex fit of microbial response and has been described in detail *(9, 10)*. van Boekel *(14)* describes the concave and convex shape response in terms of ”increasing cumulative damage” or the existence of more heat resistant sub-populations, respectively. Strong correlation

between a major parameter (the hazard function) of the Weibull model and process temperature for the majority of the data sets was evident *(14)*.

Factors which affect the shape and magnitude of thermal inactivation curves for specific microorganisms under specific conditions are not always clearly identified. Two factors affecting microbial inactivation are prior growth conditions of the organism and suspending menstruum properties at the time of exposure to heat. These factors could affect the physiological state of a particular population and add uncertainty to results from predictive models. The objective of this study was to determine if the shape and magnitude of survivor curves for the thermal inactivation of *Escherichia coli* O157:H7 could be predicted by the physiological state of the cells relative to growth conditions and heating menstruum properties at the time of heat exposure. This study allowed an extensive comparison of the commonly used log-linear model (for thermal inactivation) to the non-log-linear Weibull approach. This research can be viewed as an extension to the work presented by van Boekel *(14)*.

II. Materials and Methods

A. Inoculum. *Escherichia coli* (strain O157:H7, ATCC 43895) was grown statically under aerobic conditions (traditional method) in tryptic soy broth (TSB, Difco, Sparks, MD) for 24 h at 35°C for two consecutive transfers prior to use. This culture was also used to inoculate a chemostat vessel for controlled growth conditions.

B. Chemostat method. The chemostat enabled a control framework to achieve a quasi-constant growth rate of bacteria and nutrient concentration, allowing bacteria to stay in a sustained growth phase (*6*). The initial nutrient concentration was supplied by TSB at 27.5 g/l of deionized water. A model C30/32 BioFlo chemostat (New Brunswick Scientific, Edison, NJ) having a reaction chamber capable of producing 350 ml of cell culture was used. Product feed was maintained with a MasterFlex Peristaltic (Cole-Parmer Instrument Co., Chicago, IL) variable speed pump unit utilizing a model 7014-20 pump head (Cole-Parmer). Approximately 3-4 h following inoculation with ca. 1 ml of culture in ca. 200 ml of TSB in the growth chamber, turbidity was observed and the nutrient pump started (rate of 0.6 ml/min). Agitation in the reaction vessel was maintained at 400 rpm and filtered air introduced at ca. 0.1 liter/min. The reaction chamber was maintained at 34°C using a controlled water jacket heated by a water bath. Test culture was extracted from a sample port which collected culture from the middle of the growth chamber.

C. Heating menstrua (buffered and non-buffered). Culture from both growth methods was diluted into either a non-buffered 0.1% peptone diluent (initial pH ca. 7.0; Sigma, St. Louis, MO) or a 0.1 M phosphate buffer system adjusted to pH ca. 7.0 (Sigma).

D. Inactivation procedure. Inactivation tests were performed in duplicate for each growth condition (traditionally-grown cells, buffered and non-buffered, chemostat cells, buffered and non-buffered). Culture from the growth methods was diluted 1:10 with either 0.1% peptone diluent or pH 7.0 phosphate buffer and placed into closed screw-capped glass vials (12 x 35 mm). Initial population control counts were made on

this dilution. The total volume added to each vial was 2.1 ml. In order to minimize individual vial preparation error, the 1:10 dilution of cell culture was achieved by diluting cells into a large enough volume of buffer to fill all test vials. An open bath with a circulating heater (Haake model V26, Karlsruhe, Germany) was used to immerse and maintain two perforated aluminum baskets containing the test vials at a constant temperature prior to extraction at pre-set time intervals. The inactivation temperatures used were 58, 59, 60 and 61°C. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA). Come-up times for the vials were determined by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the center of 2 vials filled with Peptone diluent and submersed into the water bath. The thermocouples were connected to an Omega OMB-Chartscan-1400 portable data recorder utilizing ChartView Plus software (Omega Engineering). Vials of each culture preparation were extracted from the bath at prescribed time points. For both the 58 and 59°C temperatures, the initial time point was 1 min followed by 3 min and then 3 min intervals for up to 39 min. For traditionally-grown cells in peptone buffer, the time points for 60°C were performed at 1 min followed by 2 min and then 2 min intervals for up to 15 min. All remaining cell/buffer time points for 60 and 61°C were at 1 min intervals for each condition tested (up to 12 min). Upon removal, sample vials were immediately cooled in an ice water bath to stop further thermal inactivation.

E. Enumeration of survivors. After the vials were cooled, samples were taken for enumeration of survivors within 30-40 min. The earlier time points were serially diluted as needed to achieve readable plates for enumeration. Each replication was plated on

tryptic soy agar (TSA, Difco) in duplicate using a spiral plater (Don Whitley Scientific Limited, Yorkshire, UK). Plates were incubated for 48 h at 35°C before enumeration of *E. coli* O157:H7 using a Protocol automatic plate counter (Synoptics Limited; Cambridge, UK). For later time points, where specific populations were below log 3 CFU/ml, undiluted samples were spread plated (0.4 ml) on TSA plates for more precise counts.

F. Curve fitting. Regression analysis was performed using the GInaFiT freeware tool (Source?)for Microsoft® Excel (*4*), as well as the standard curve fitting capabilities of Excel. This regression package is capable of both linear and non-linear approaches, with and without tail effects. Two of the nine models describing microbial survival in the GInaFiT tool were used in the analysis of these data: 1) the model presented by Bigelow and Esty in 1920, as described by Geeraerd et al. (*4*) for log-linear response, (which has the functional form):

$$\log_{10}\left(N(t)\right) = \log_{10}\left(N(0)\right) - \left(\frac{t}{D}\right)$$

where *N(t)* is the observed population at time *t* of the process and *N(0)* is the initial population (*D* (-value) is the time for a 90% population reduction at the isothermal process temperature), and 2) the Mafart et al. (*8*) model, which uses the non-linear Weibull function, either with or without a fixed shape factor: (which has the functional form):

$$\log_{10}\left(N(t)\right) = \log_{10}\left(N(0)\right) - \left(\frac{t}{\delta}\right)^{p}$$

where δ is the hazard rate (time of first decimal reduction) and p is the shape factor. In addition to the fit statistics provided by the freeware tool, a 'goodness of fit' tool recommended by several researchers (*1, 2, 7*) was used. It is a mathematical technique that relates the percentage of variance (%V) accounted for by the model (based on number of terms):

$$\%V = \left[1 - \frac{(1-r^2)(n-1)}{n - N_T - 1}\right] \times 100$$

Where r is the correlation coefficient, n is the number of data points and N_T is the number of model equation terms. This coefficient takes into account the complexity of the model and the population of data used to describe it.

G. Response magnitude. Data were analyzed with both the log-linear and Weibull methods and the time to a specific decline in population was compared. The equation for determining the time to a specific log reduction for the Weibull function *(14)* is given by:

$$t_d = \delta\left(-\ln\left(10^{-d}\right)^{\frac{1}{p}}\right)$$

where d is the number of decimal reductions. This equation is based on the "characteristic time" (90% reduction) of the Weibull function and can be used as a comparison to D-value calculations. The benefit of this equation over the standard log-linear calculation is that it accounts for the shape, as well as magnitude, of the inactivation curve.

H. Parameter analysis. Temperature dependence of the D-value is described using the z-value (the temperature change necessary for a 90% change in D-value or a 1 log shift in log D-value)(*13*). Likewise, van Boekel *(14)* showed the temperature dependence of the hazard function (δ) in Weibull applications. The shape factor (p) has not generally been shown to be temperature dependent for microbiological data. Graphical comparisons of model parameters versus process temperature are given for log D-value (z-value), the Weibull hazard rate (δ), and shape factor (p).

III. Results and Discussion

Traditionally-grown or statically incubated cells in culture tubes likely followed a more anaerobic-fermentative pathway, as evidenced by a reduction of the pH of the growth medium from ca. 7.2 to ca. 6.25. The chemostat cells, which were exposed to constant mixing and introduction of air, likely followed an aerobic pathway and the pH of the growth media actually increased to ca. 8.1. It has been suggested that *E. coli* in fed-batch cultures produce less organic acids as by-products when glucose is maintained at levels lower than needed for maximum growth. Researchers have also noted the presence of higher concentrations of ammonium and nitrates in such cultures, which results in higher culture pH (*11*). Corresponding pH of traditionally-grown and chemostat cells, when diluted into 0.1% peptone buffer for thermal inactivation tests were ca. 6.6 and ca. 7.6, respectively. With the phosphate buffer system, the final pH of

traditionally-grown cells was 6.95-7.05, while the chemostat cells had a slightly higher pH range of 7.02-7.18.

Figures 6 through 9 show the inactivation response for each growth condition and pH heating menstruum combination for the temperatures in this study. The last data point for all combinations is that in which consistent recovery of countable colonies was achieved. Figure 6 shows traditionally-grown cells heated in peptone buffer. There was a strong concave downward response, denoting decreasing heat resistance with time, with no overlapping data between process temperatures. When comparing chemostat cells heated in peptone buffer (Fig. 7) to traditionally-grown cells, there were distinct differences in magnitude and shape response. The chemostat cells showed a concave upward shape which denoted increasing thermal resistance at later time points. Chemostat cells showed a significant increase in thermal sensitivity relative to the points where the last surviving cells were enumerated (from 18 to 5 min, for 58 to 61°C, respectively). The corresponding times at which the last surviving cells were detected for traditionally-grown cells in peptone buffer was considerably longer, from 37 to 7 min, for 58 to 61°C (Fig. 6). Greater variability or data scatter was noticed in peptone buffer for both growth types relative to phosphate buffer. Humpheson et al. (*6*) compared heat inactivation of statically grown *Salmonella* Enteritidis (PT4) to those grown in a chemostat and found no significant differences. However, the researchers buffered the feed medium (pH 6.5), instead of the heating menstruum, and introduced cells into a preheated test solution.

Responses changed when the heating menstruum was phosphate buffer (pH 7.0) for both growth types (Fig. 8 and 9). The magnitude of response for traditionally-grown cells changed considerably and the shape and variability of the data were affected for both growth types. Figure 8 illustrates a more log-linear inactivation response for traditionally-grown cells in phosphate buffer at 58°C, while the remaining temperatures retained a concave shape. The magnitude of response for traditionally-grown cells in phosphate buffer was lower at 58°C (when compared with cells heated in peptone) and was not significantly different to chemostat cell response for the remaining temperatures (59-61°C). Chemostat cells in phosphate buffer (Fig. 9) showed a more linear response for all process temperatures and could be effectively modeled with either the log-linear or Weibull methods. The variability of recovery at each time point (data scatter) was less for both growth methods in the phosphate buffered system. This is possibly due to a more uniform environment with respect to pH.

Table 2 shows the number of data points (n), the parameters for both the log-linear and Weibull models, and the fit statistics for all conditions tested. More data points were observed for traditionally-grown cells in peptone due to the longer inactivation times (i.e., greater thermal resistance) for these cells. The most statistically significant 'goodness of fit' difference between the log-linear and Weibull models was seen for tradition cells in peptone at 58°C, with percent variance accounted for by the model (%V) values of 83.37 (R^2=0.84) compared to 98.41% (R^2=0.99), respectively. These correlation values indicate the Weibull model is the more accurate for prediction purposes. The Weibull shape factors for traditional cells for all but one condition (58°C

in phosphate buffer) were greater than 1, indicating a concave downward shape. When traditionally-grown cells were treated in phosphate buffer at 58°C, the log-linear model was just as accurate as the Weibull model (shape factor of 1.0), with %V values of 98.46 (R^2=0.99) and 98.43% (R^2=0.99), respectively. The %V and R^2 values were equivalent in most cases, except at 61°C for traditional cells in phosphate buffer where the %V values were approximately 8 to 10% lower than R^2 values. This difference resulted from the lower number of observations (n=5 for one replication; total n=21) for this test condition, which reduced the relative 'goodness of fit' in the %V approach. The log-linear and Weibull models showed comparable 'fit statistics' for chemostat cells in almost all process conditions (except in peptone at 58°C, where the %V values were 90.83% for log-linear vs. 98.51% for Weibull). Weibull shape factor values were equal to or less than 1 for all chemostat-grown cells, indicating concave upward shape and consequently increasing thermal resistance of the populations. It is important to note the shape factors were > or equal to 1 (concave downward) for traditionally-grown cells. When comparing either the log-linear D-value or the non-log-linear Weibull hazard rate (δ), the overall magnitude of resistance for traditional cells in peptone was twice that of chemostat cells in either heating menstruum. Therefore, traditionally-grown cells (in all but one instance) showed increasing susceptibility to heat during the process, while chemostat cells showed increasing resistance. It is possible that the mildly acidic conditions of static growth contributed to population resistance in comparison to the relatively stress free conditions of the chemostat. Peleg and Cole (*10*) hypothesized that

a microbial population's underlying sensitivities and resistances are best described in the form of distribution that does not always obey the first-order kinetics model.

Figure 10 shows the results for heat inactivation at 58°C for both the traditionally-grown and chemostat cells. The magnitude and shape differences between traditionally-grown and chemostat cells were more distinguishable at this temperature. It took approximately twice as long (36 min vs. 18 min) to fully inactivate traditionally-grown cells (initially ca. 8 log CFU/ml) compared with chemostat cells (initially ca. 9 log CFU/ml) in peptone.

The results in this study show stronger log-linear response for chemostat cells and traditionally-grown cells in phosphate buffer, with R^2 values above 0.98 for both. The non-log-linear Weibull function more accurately approximates the shape of traditionally-grown cells (R^2=0.99) and chemostat cells (R^2=0.99) in peptone. The curvilinear shape of traditionally-grown cells in peptone became less pronounced as temperature increased.

A comparison of death time calculations between the log-linear and Weibull models for all test conditions is shown in Table 3. An effective means of comparing magnitude response between the log-linear and Weibull models is the calculation of the time to a specific log reduction, as demonstrated by van Boekel *(14)*. The shape factor (*p*) of the Weibull model is important when comparing predicted time points of specific log reductions to those of the log-linear model. With traditionally-grown cells in peptone or phosphate buffer, the concave downward response resulted in more conservative time estimates for a 5-log reduction and generally less conservative

estimates for an 8-log population reduction using the Weibull compared to the log-linear model. The more conservative estimates for 5-log reduction times were seen for traditionally-grown cells in peptone, where relative inactivation times from the Weibull model range from ca. 14 to 22% percent higher than predictions from the log-linear model. For 8-log reductions, predicted times from the Weibull model were less conservative by as much as 14% (37.41 min (Weibull) vs. 43.92 (log-linear) for traditionally-grown cells in peptone at 58°C). The opposite was true for chemostat cells in peptone or phosphate buffer where the concave upward shape resulted in less conservative time estimates for a 5-log reduction and more conservative time estimates for a 9-log reduction. Chemostat populations (log CFU/ml) were approximately 1 log higher compared with traditionally-grown cells, likely as a result of optimal growth conditions.

Figure 11 gives a graphical view of log D vs. process temperature, allowing calculation of z-values for each growth type/menstruum combinations. Traditionally-grown cells showed a more significant shift in z-value from peptone to phosphate buffer, 3.97 to 3.18°C, while chemostat cells showed less of a difference, 4.61 to 4.84°C, respectively. The downward shift in z-value (-0.79°C) for traditionally-grown cells in peptone or phosphate buffer demonstrated an increase in thermal sensitivity for this growth type when buffered to a more neutral pH. Conversely, chemostat cells started from a higher pH in peptone (ca. pH 7.6) and did not show a significant change in resistance when adjusted to ca. pH 7. Temperature dependence has also been shown for the Weibull hazard rate (δ) *(14)*. Figure 12 represents the graphical correlation of the

log hazard rate to process temperature. Traditionally-grown cells, in peptone and phosphate buffer, showed consistent temperature dependence. Traditionally-grown cells in phosphate buffer showed a less dramatic change (log δ of 0.57 to 0.28), while there was a significant shift for peptone cells (log δ of 1.27 to 0.39). These significant differences in log δ (for traditionally-grown cells in peptone) are confirmed graphically in the magnitude shifts seen in Table 2. It was more difficult to characterize temperature dependence of the hazard rate (δ) for chemostat cells, due to their higher variability (data scatter) and lower magnitude of response. The thermal resistance for chemostat cells was relatively low compared to traditionally-grown cells (regardless of heating menstruum) and did not change consistently with temperature.

Figure 13 shows the relationship of the Weibull shape factor (log p) to process temperature. Traditionally-grown cells in phosphate buffer showed the strongest correlation with temperature. As pointed out earlier in graphical comparisons, buffering traditionally-grown cells to pH 7 at 58°C resulted in a nearly log-linear response which became more concave as the temperature increased. The alternate was true for traditionally-grown cells in peptone, where the shape factor (p) showed a shift away from stronger concavity with increasing temperature (log p of 0.48 to 0.29). Chemostat cells did not show strong correlation between shape factor and process temperature for either heating menstruum. A similar lack of correlation for the Weibull shape factor with process temperature was observed by van Boekel *(14)*.

In conclusion, results suggest differences in the inactivation response of microbial cells depending on their growth state and heating menstruum at the time of

inactivation. The results in this study also suggest the Weibull function offers more flexibility in analyzing the inactivation characteristics of *E. coli* O157:H7 under controlled conditions, which supports the conclusions of previous researchers *(8, 10, 14)*. Higher thermal resistance for traditionally-grown cells could indicate cross protection because of a mild acid shock response (pH 6.25 in growth media) in addition to growth condition effects, however further experiments would be needed to confirm this.

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Appendix

Table 2 Model parameters and 'Fit Statistics' for log-linear and Weibull methods

Temp (ºC)	n[1]	D-value	log D	δ[2]	p[3]	Linear %V[4]	Weibull %V	Linear R^2	Weibull R^2
Traditionally-Grown Cells in Peptone									
58	108	5.49[5]	0.74	18.67	3.01	83.37	98.41	0.84	0.99
59	74	3.08	0.48	7.64	1.90	92.04	97.62	0.93	0.98
60	52	1.48	0.17	3.45	1.76	91.30	96.54	0.92	0.97
61	52	0.85	0.00	2.47	1.94	93.26	98.74	0.94	0.99
Mean						**89.99**	**97.83**	**0.91**	**0.98**
SD[6]						**4.49**	**0.98**	**0.04**	**0.01**
Traditionally-Grown Cells in Phosphate Buffer, pH 7.0									
58	73	4.00	0.60	3.68	1.00	98.46	98.43	0.99	0.99
59	32	1.55	0.19	3.29	1.59	91.74	97.35	0.93	0.98
60	30	0.88	-0.06	2.48	2.75	92.15	98.90	0.94	0.99
61	21	0.44	-0.36	1.89	3.06	78.89	88.17	0.89	0.96
Mean						**90.31**	**95.71**	**0.93**	**0.98**
SD						**8.21**	**5.07**	**0.04**	**0.02**
Chemostat-Grown Cells in Peptone									
58	68	2.23	0.35	0.13	0.49	90.83	98.51	0.92	0.99
59	43	1.42	0.15	0.46	0.69	98.17	98.66	0.98	0.98
60	43	0.88	-0.06	0.04	0.38	94.36	93.16	0.96	0.96
61	35	0.50	-0.31	0.02	0.41	96.27	97.24	0.97	0.98
Mean						**94.91**	**96.89**	**0.96**	**0.98**
SD						**3.13**	**2.57**	**0.03**	**0.01**
Chemostat-Grown Cells in Phosphate Buffer, pH 7.0									
58	34	1.78	0.25	0.75	0.73	98.11	99.09	0.98	0.99
59	32	1.15	0.06	0.71	0.84	98.17	98.66	0.99	0.99
60	34	0.65	-0.19	0.09	0.57	95.19	96.24	0.96	0.97
61	24	0.44	-0.36	0.44	1.00	99.12	99.07	0.99	0.99
Mean						**97.65**	**98.26**	**0.98**	**0.99**
SD						**1.70**	**1.36**	**0.01**	**0.01**

[1]n = number of observations
[2]δ = the Weibull hazard function
[3]p = the Weibull shape factor
[4]%V = the % variance accounted for by the model
[5]log-linear approach is statistically less accurate than Weibull for this case
[6]Standard Deviation

Table 3 Comparison of time to specific log reduction of log-linear vs. Weibull

Temp (ºC)	5-D Linear (min)	5-D Weibull (min)	8-D Linear (min)	8-D Weibull (min)	% Diff[1] 5-D	% Diff 8-D
Traditionally-Grown Cells in Peptone						
58	27.45	31.96	43.92	37.41	16.48	-14.78
59	15.38	17.85	24.60	22.90	16.94	-5.91
60	7.40	8.92	11.84	11.89	21.87	0.70
61	4.25	5.67	6.80	7.22	33.31	20.02
Traditionally-Grown Cells in Phosphate Buffer, pH 7.0						
58	20.00	18.42	32.00	29.47	-7.92	-7.90
59	7.75	9.06	12.40	12.18	16.89	-1.78
60	4.40	4.60	7.04	5.53	4.75	-21.20
61	2.18	3.23	3.48	3.78	49.94	9.91
Chemostat-Grown Cells in Peptone						
58	11.13	8.29	20.03	27.82	-25.14	39.49
59	7.10	6.78	12.78	15.92	-4.59	24.59
60	4.30	3.34	7.74	10.34	-22.40	33.53
61	2.48	1.07	4.46	4.40	-57.62	-1.69
Chemostat-Grown Cells in Phosphate Buffer, pH 7.0						
58	8.90	9.23	16.02	20.74	3.71	29.52
59	5.73	5.52	10.31	11.34	-3.62	10.00
60	3.25	2.84	5.85	7.97	-12.58	36.22
61	2.20	2.20	3.96	3.96	-0.04	-0.03

[1]% Difference based on Weibull versus log-linear method

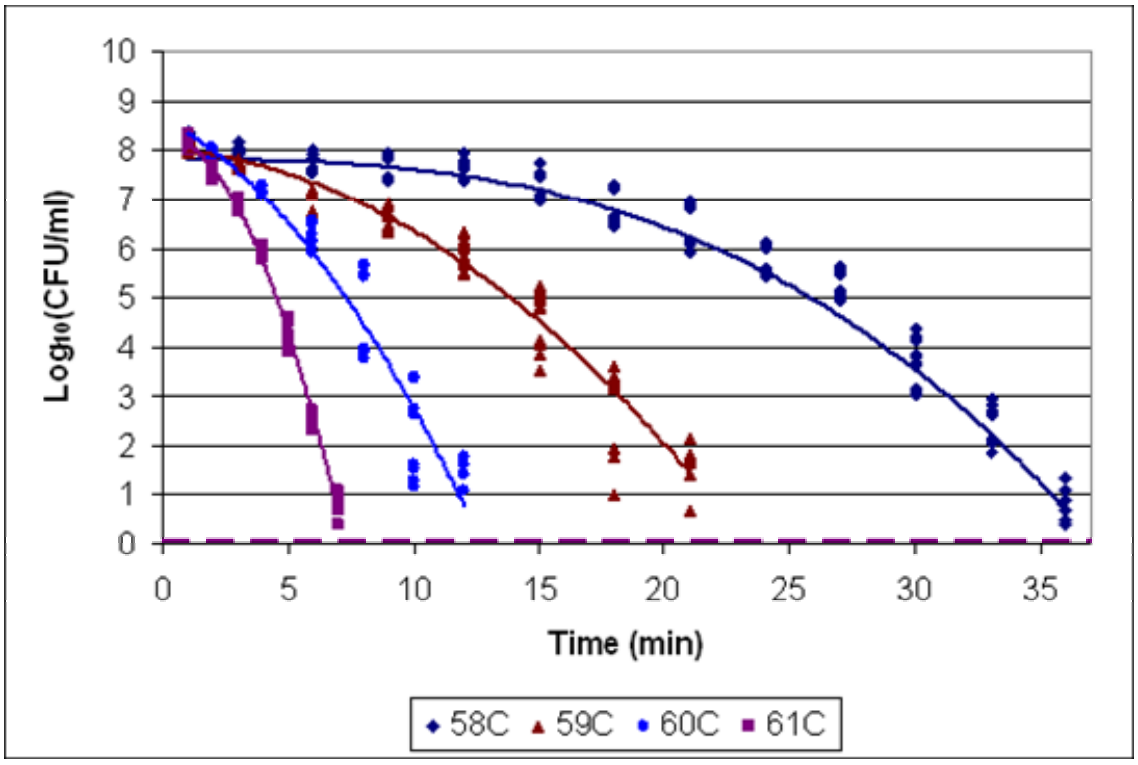

Figure 6 Inactivation of *Escherichia coli* O157:H7 *in vitro* from 58 to 61°C, grown 24 h under static conditions, ~ pH 6.25 during growth and ~ pH 6.6 (in peptone) during heating, using the Weibull model

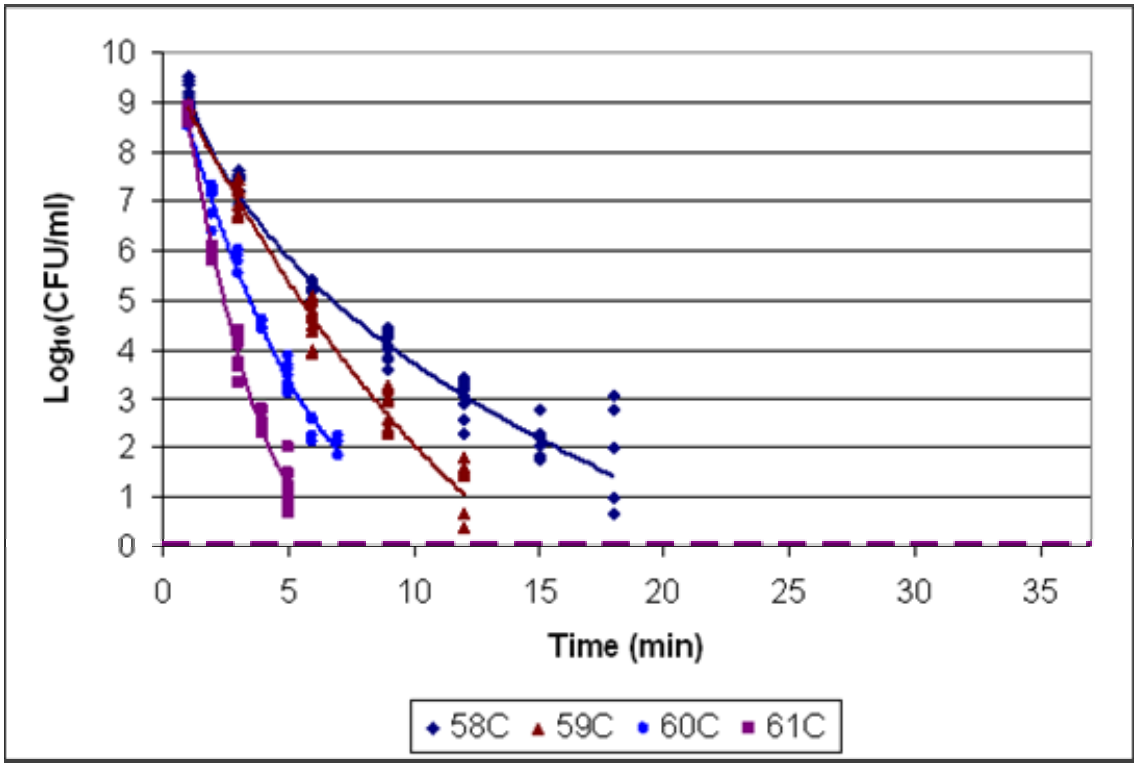

Figure 7 Inactivation of *Escherichia coli* O157:H7 *in vitro* from 58 to 61°C, grown in a chemostat, ~ pH 8.1 during growth and ~ pH 7.6 (in peptone) during heating, using the Weibull model

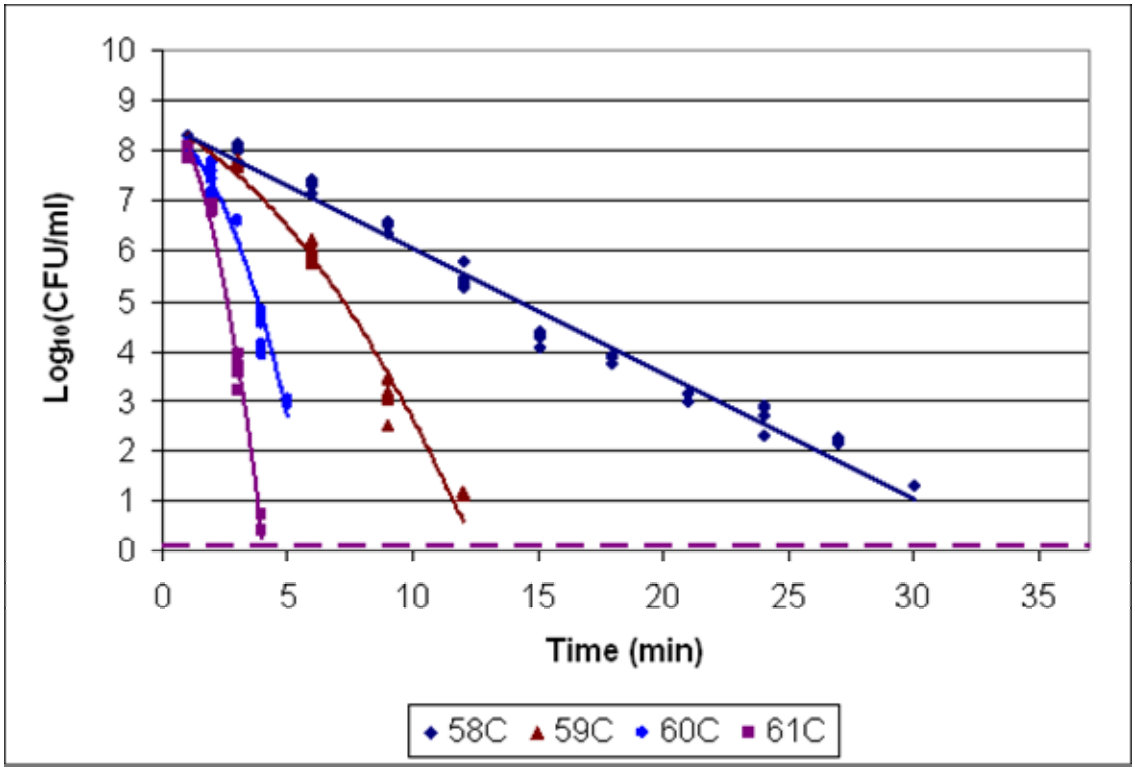

Figure 8 Inactivation of *Escherichia coli* O157:H7 *in vitro* from 58 to 61°C, grown 24 h under static conditions, ~ pH 6.25 during growth and ~ pH 7.0 (in phosphate buffer) during heating, using both the log-linear and Weibull models

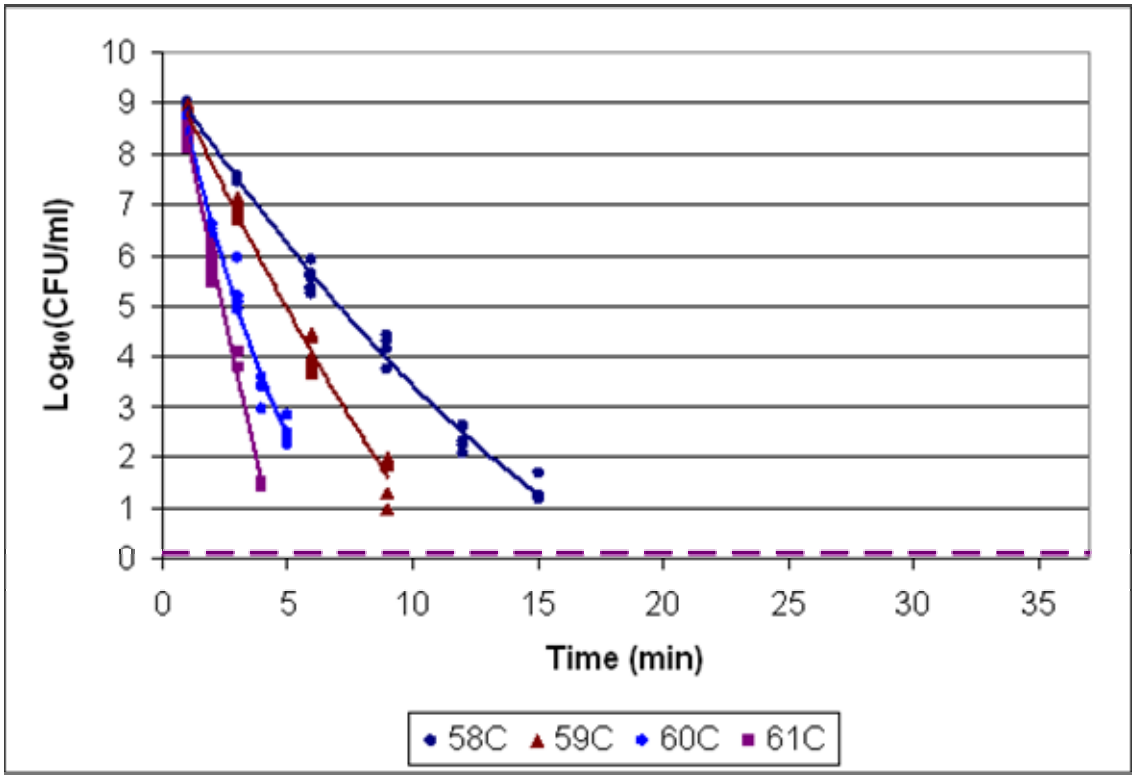

Figure 9 Inactivation of *Escherichia coli* O157:H7 *in vitro* from 58 to 61°C, grown in a chemostat, ~ pH 8.1 during growth and ~ pH 7.1 (in phosphate buffer) during heating, using the Weibull model

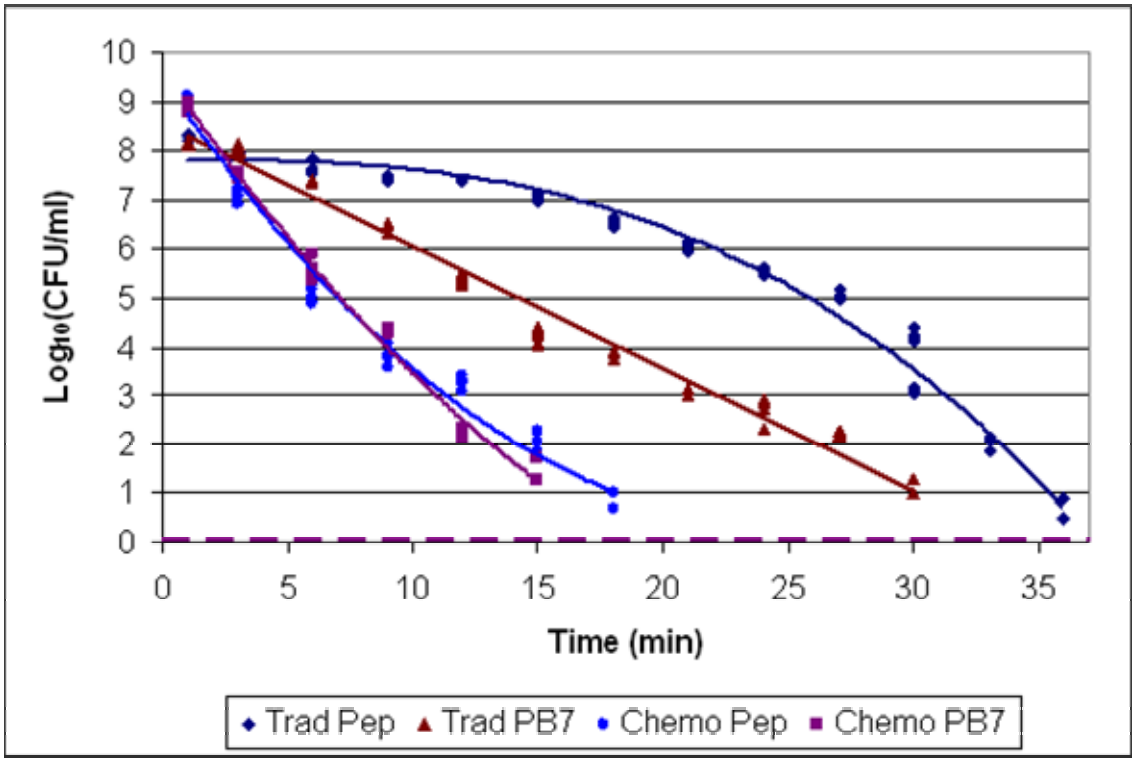

Figure 10 Inactivation Comparison of *Escherichia coli* O157:H7 *in vitro* at 58°C, grown by traditional method and in a chemostat, in peptone (Pep) and phosphate buffer (PB7), using both the log-linear and Weibull models

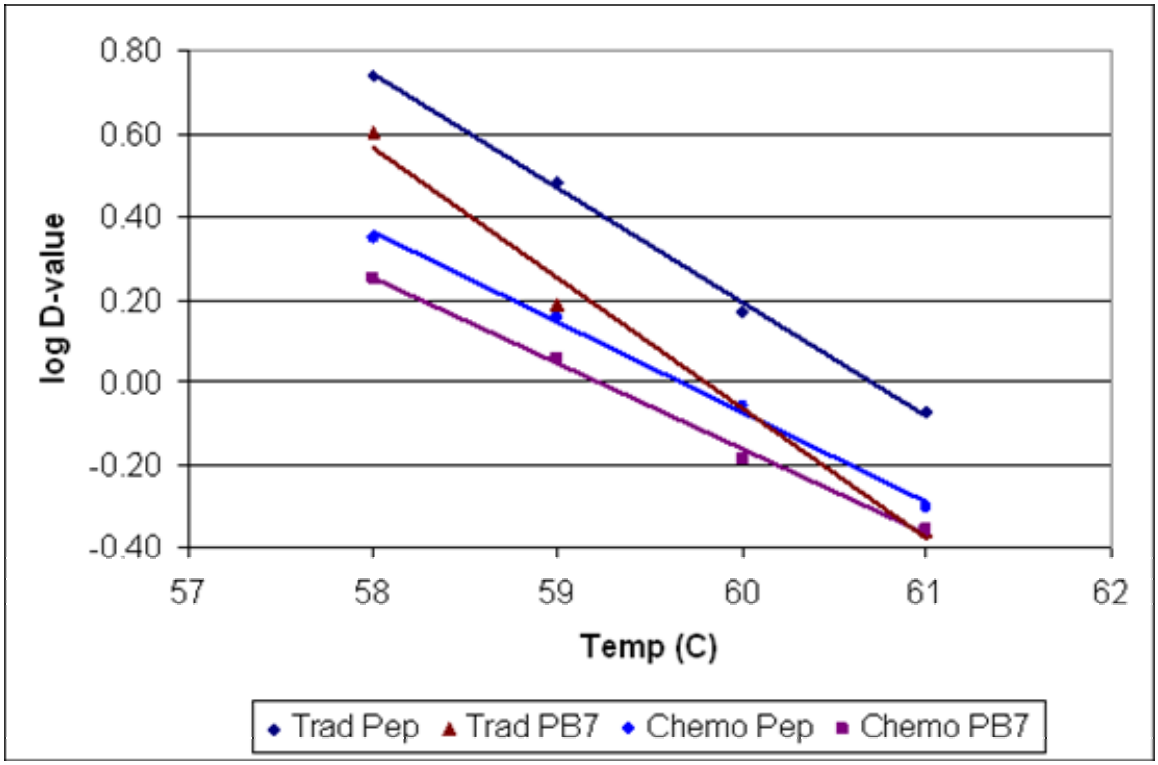

Figure 11 log D-value versus Process temperature (z-value graph) for *Escherichia coli* O157:H7 grown by traditional method and in a chemostat, in peptone (Pep) and phosphate buffer (PB7)

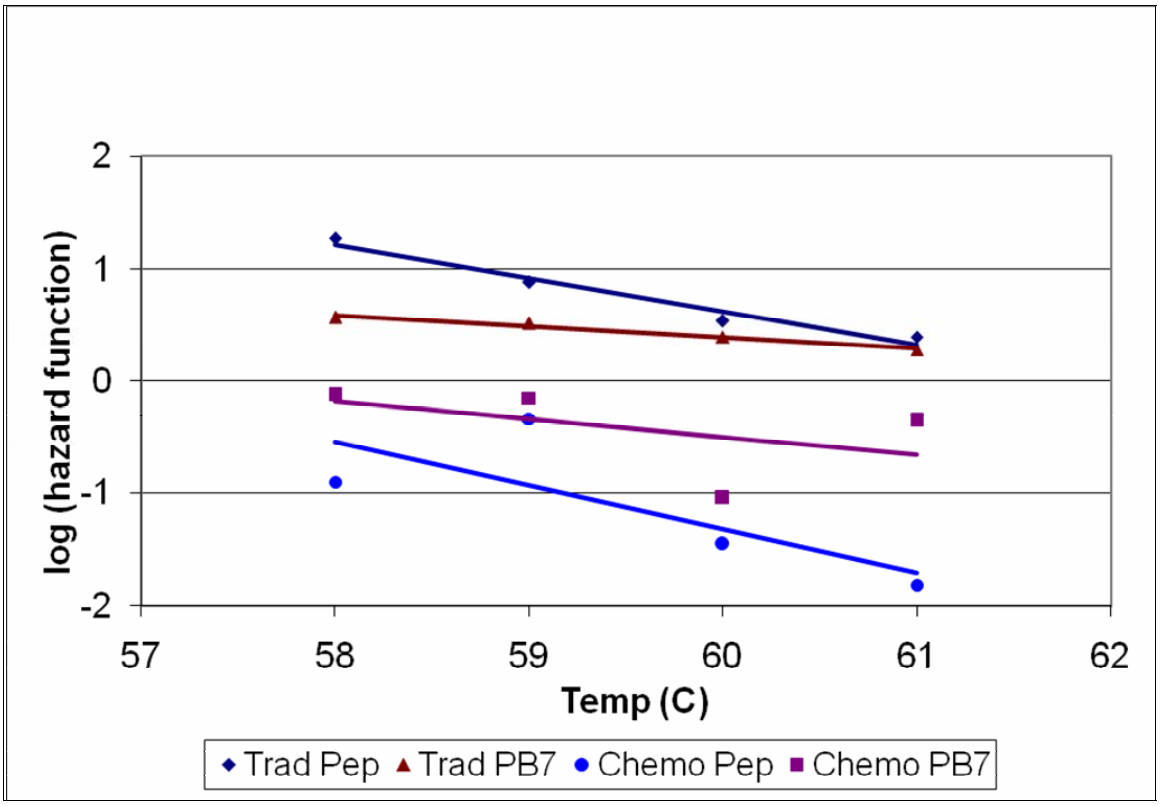

Figure 12 Weibull hazard rate function (δ) versus Process temperature for *Escherichia coli* O157:H7 grown by traditional method and in a chemostat, in peptone (Pep) and phosphate buffer (PB7)

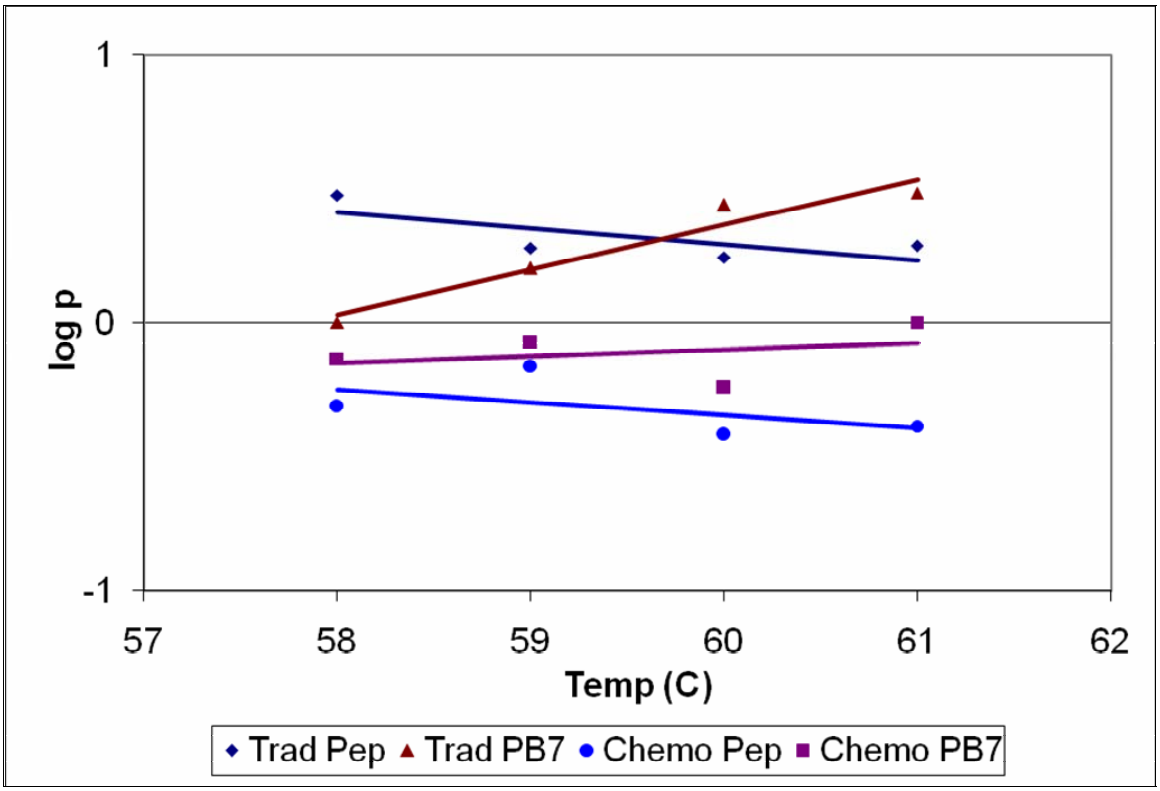

Figure 13 Weibull shape factor (p) versus Process temperature for *Escherichia coli* O157:H7 grown by traditional method and in a chemostat, in peptone (Pep) and phosphate buffer (PB7)

PART FOUR: DEVELOPMENT OF A SIMULATED BEEF BROTH SYSTEM FOR THERMAL INACTIVATION ANALYSIS OF *ESCHERICHIA COLI* O157:H7

Abstract

The environmental conditions in which a microbial cell is suspended at the time of thermal inactivation play a significant role in the characteristic resistance response of the cell. It is important from the standpoint of cost and time management to model real food systems in order to predict microbial response in specific products. The objective of this study was to compare the heat resistance of *Escherichia coli* O157:H7 in a simulated beef broth system to the free liquid filtrate of raw ground beef. *E. coli* O157:H7 ATCC 43895 was grown aerobically in a static incubator. Heating menstrua included both buffered and non-buffered laboratory diluent, a simulated beef broth and actual beef broth obtained from 93% lean ground beef. Survivor curves were prepared by heating cultures in vials at 60, 61 and 62°C for up to 20 min and recovering survivors on tryptic soy agar. A regression package capable of both linear and non-linear approaches was used to analyze results. There were significant differences in D-values when cells were thermally treated in either simulated or actual beef broth mixtures compared to peptone or phosphate diluents (from 17 to 42% higher, respectively). D_{61C} values for simulated and actual beef broths were 1.1 and 1.13 min compared to 0.85 and 0.91 min for peptone and phosphate, respectively. D and z-value results for actual beef broth were similar to those found in the literature for combined meat studies. Results suggested that a simulated beef broth system could provide similar information to actual beef broth in determining the thermal inactivation characteristics of *E. coli*

O157:H7. The use of the Weibull model offered more flexibility and accuracy of fit when compared to the traditionally used log-linear approach for the data in this experiment.

I. Introduction

The environmental conditions in which a microbial cell is suspended at the time of thermal inactivation play a significant role in the characteristic resistance response of the cell. A number of review articles have discussed the influence of heating menstruum on the heat resistance of various foodborne pathogens (*1, 2, 10, 11*). Significant response differences have been shown between thermal resistance in actual food components and laboratory media.

Factors that affect the shape and magnitude of the thermal resistance response of a microorganism under specific conditions are not always clearly defined. Intrinsic factors associated with the food that affect the survival and inactivation of microorganisms have been explored (*4, 8, 14*). For example, Geeraerd et al. (*4*) described how proteins and fats can add to thermal tolerance of microorganisms. They suggested that proteins may assist in preventing the loss of solutes, stabilizing the cell membrane and providing a buffering effect against lower pH conditions. Further, they proposed that fat molecules effectively reduced water activity around the cell resulting in lower thermal delivery (*4*).

It is important from the standpoint of cost and time management to model real food systems in order to predict microbial response in specific products. Ground beef presents a considerable challenge to the researcher wishing to model microbial inactivation due to its various constituents, i.e. peptides, free amino acids and glycogen (*3*). While some researchers have studied the effects of different fat levels on thermal

inactivation of microorganisms in meat products (*8*), little focus has been placed on the free liquid portion of ground meats. The objective of this study was to determine the influence of the free liquid filtrate of raw ground beef on the heat resistance of *E. coli* O157:H7 and to develop a simulated beef broth system (based on previous work by Juneja et al. (*7*)) to model similar inactivation studies in the laboratory. A comparison of the non-log-linear Weibull regression method to the log-linear model for thermal inactivation was also conducted. A number of researchers have discussed the use of non-log-linear models to describe thermal death curves for both the inactivation of vegetative cells and spores (*5, 6, 13*).

II. Materials and Methods

A. Inoculum. *E. coli* O157:H7 ATCC 43895 was inoculated into tryptic soy broth (TSB, Difco, Sparks, MD) in screw-capped test tubes and grown statically in an aerobic atmosphere for 24 h at 35°C for two consecutive transfers prior to use in thermal inactivation studies.

B. Simulated beef broth. A simulated beef broth was developed to model the free liquid filtrate of raw ground beef (i.e., the portion which remains liquid during the cooking process). The simulated beef broth system (SimBB) was modified from a model beef gravy system used by Juneja et al. (*7*). The SimBB contained 0.15% (w/v) proteose peptone (Sigma, St. Louis, MO), 0.5% (w/v) beef extract (Difco) and 0.05% (w/v) yeast

extract (Difco). The pH of simulated beef broth was adjusted to ca. 5.9 with a 0.1M phosphate buffer system.

C. Beef broth. Fresh ground beef (93% lean) was obtained from a local market and the free liquid filtrate (beef broth, BB) was extracted by initially manually pressing the liquid through a cheese cloth. Additional liquid was then removed by centrifugation at 2000 x G (Beckman J2-HS Centrifuge, Fullerton, CA) for 5 min, followed by collection of the supernatant. The extracted broth was heated in a laboratory pressure vessel to 121.6°C to facilitate coagulation and gelling of free proteins in the liquid. The liquid was vacuum filtered to remove remaining particulates using a ceramic laboratory funnel and a 110 mm diameter paper filter (Whatman, Cat. No.1001-110, Maidstone, England). The liquid was sterilized by membrane filtration using a 0.22 µm low protein binding cellulose acetate filter (Corning, Corning, NY).

An infrared spectral analysis was performed on the BB and SimBB using Fourier transform infrared (FTIR) analysis with attenuated total reflection infrared (ATR) accessory (Nexus 680, Thermo Nicolet Corp, Madison, WI) to compare the relative composition of each (Appendix).

D. Heating menstrua. Culture was diluted (1:10) into non-buffered 0.1% (w/v) peptone diluent (Sigma, initial pH ca. 7.0) (PEP), 0.1 M phosphate buffer (Sigma) adjusted to pH 5.9 (PB59), simulated beef broth (SimBB), and a non-buffered free liquid filtrate of ground beef (BB).

E. Inactivation procedure. Thermal Inactivation tests were performed in duplicate for each heating menstruum. The diluted culture/menstruum was placed into

closed screw-capped glass vials (12 x 35 mm). Initial population control counts were made on this dilution. The total volume added to each vial was 2.1 ml. In order to minimize individual vial preparation error, the 1:10 dilution of cell culture was achieved by diluting cells into a large enough volume of the respective heating menstrua to fill all test vials. An open bath equipped with a circulating heater (Haake model V26, Karlsruhe, Germany) was used to immerse and maintain a perforated aluminum basket containing the test vials at a constant temperature. The inactivation temperatures tested were 60, 61 and 62°C. Water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA). Come-up times for the vials were determined by placing type-T thermocouples (Omega Engineering, Inc., Stamford, CT) in the center of 2 vials filled with peptone diluents and submerged into the water bath. The thermocouples were connected to portable data recorder (OMB-Chartscan-1400, Omega) utilizing ChartView Plus software. Vials of each culture preparation were removed from the bath at prescribed time points. For 60°C, the initial time point was 1 min followed by 2 min and 2 min intervals for a total time of 20 min, for all conditions. All time points for 61 and 62°C were at 1 min intervals for each condition tested (up to 12 min). Upon removal, sample vials were immediately cooled in an ice water bath.

F. Enumeration of survivors. After the vials were cooled, surviving cells were enumerated within 30 - 40 min. For earlier time points, samples were serially diluted as needed to achieve readable plates for enumeration. Each replication was plated on tryptic soy agar (TSA, Difco) in duplicate using a spiral plater (Don Whitley Scientific Limited, Yorkshire, UK). Plates were incubated for 48 h at 35°C before enumeration of *E.*

coli using a Protocol automatic plate counter (Synoptics Ltd., Cambridge, UK). For later time points, where populations were below 3 log CFU/ml, undiluted samples were spread plated (0.4 ml x 2 sub-samples per vial) on TSA plates for more precise counts.

G. Curve fitting. Regression analysis was performed using the GInaFiT freeware tool for Microsoft® Excel (*5*), as well as the standard curve fitting capabilities of Excel. Geeraerd et al. (*5*) created a freeware Add-in for Microsoft® Excel (GInaFiT) which bundled a group of static models capable of fitting 8 common shapes found in microbial inactivation data.

The user enters experimental data relating time and log population counts and is given the choice of both linear and non-linear approaches, with and without tail effects. The authors give the model source and basic governing equations and the output includes parameter estimates with standard error, overall model Sum of Squared Errors (SSE), Mean Sum of Squared Errors (MSE), and Root Mean Sum of Squared Errors (RMSE). Two of the nine models describing microbial survival in the GInaFiT tool were used in the analysis of this data: 1) the model presented by Bigelow and Esty in 1920 for log-linear response (which has the functional form):

$$\log_{10}\left(N(t)\right) = \log_{10}\left(N(0)\right) - \frac{t}{D}$$

where *N(t)* is the observed population at time *t* of the process and *N(0)* is the initial population (*D* -value is the time for a 90% population reduction at the isothermal process temperature), and 2) the Mafart et al. (*9*) model, which uses the non-linear

Weibull function, either with or without a fixed shape factor: (which has the functional form):

$$\log_{10}(N(t)) = \log_{10}(N(0)) - \left(\frac{t}{\delta}\right)^{p}$$

where δ is the hazard rate (time of first decimal reduction) and p is the shape factor. Fixing the Weibull shape factor (p) to a value of 1.0 makes the hazard rate function mathematically equivalent to the D-value.

H. Response magnitude. Data were analyzed with both the log-linear and Weibull methods and the time to a specific decline in population was compared. The equation for determining the time to a specific log reduction for the Weibull function (*12*) is given by:

$$t_d = \delta\left(-\ln\left(10^{-d}\right)^{\frac{1}{p}}\right)$$

where d is the number of decimal reductions. This equation is based on the 'characteristic time' (90% reduction) of the Weibull function and can be used as a comparison to D-value calculations. A 4 log reduction level was chosen as the likely point of maximum difference between the 2 methods. A 7 log reduction level was chosen for the higher level of inactivation. Percent difference (%Diff) values were based on the Weibull time-to-reduction values divided by the corresponding log-linear values (with positive values denoting longer reduction times calculated for the Weibull method).The benefit of this equation over the standard log-linear calculation is the fact it accounts for the shape, as well as slope magnitude, of the inactivation curve.

III. Results and Discussion

When *E. coli* O157:H7 was grown in TSB, the culture likely followed a more anaerobic, fermentative pathway in the static incubator as evidenced by a lowering of the culture pH from an initial ca. 7.2 to ca. 6.25 at stationary phase. When diluted into 0.1% peptone (initial pH ca. 7), the pH recovered to ca. 6.6. The pH for the phosphate buffer system (PB59), SimBB and BB were unchanged from their original preparation after addition of the inoculum (pH ca. 5.9, 5.9 and 6.0, respectively).

Figure 14 shows the heat inactivation curves for *E. coli* O157:H7 in all menstrua at 60°C. Results for cells in PB59 and SimBB showed a more linear response. Cells in PEP and BB showed a non-linear response (concave downward). These observations were confirmed by Weibull shape factors (Table 4) of 1.33 and 1.28 for PB59 and SimBB, respectively, compared with values of 1.76 for PEP and 1.59 for BB. For the Weibull method, a shape factor closer to 1 denotes a more linear response. The concave downward shape suggests an increasing susceptibility to heat for *E. coli* O157:H7 cells as the thermal process progressed (*12*). Cells heated in SimBB required a longer inactivation process, showing higher D_{60C} values (2.12 min) when compared to the other heating menstrua (Table 4). The highest variability, or data scatter, was seen in BB at the 12 min point (stdev = 0.85). It is important to note the D-value (log-linear) approach lacks accuracy for the analysis of cells which display non-log-linear inactivation curves (in this study). D-values are given here as a means of comparison (magnitude response).

At 61°C (Fig. 15) there were no significant differences ($P < 0.05$) in response between cells in SimBB and BB. Cells heated in the BB menstruum did retain a concave

downward shape, but the overall magnitude of response was comparable to that in SimBB (with D_{61C} values of 1.10 min for SimBB and 1.13 min for BB, Table 4). Cells heated in PEP also retained a concave downward shape, but showed significantly lower thermal resistance (D_{61C}=0.85 min) compared with SimBB and BB. Thermal inactivation data for cells in PB59 (at 61°C) showed higher variability (scatter) and were not statistically different ($P < 0.05$) from the other heating menstrua thru 7 min.

A significant separation of inactivation responses was seen at 62°C between cells in diluents vs. beef broth menstrua (Fig. 16). There was no statistical difference ($P < 0.05$) in cell response between PEP and PB59. From Table 4, the D_{62C} values for PEP and PB59 were similar (0.48 and 0.51 min, respectively). There was a similar grouping of the inactivation curves for cells in SimBB and BB at this temperature, with no significant differences ($P < 0.05$) in cell response between the 2 menstrua. D_{62C} values for SimBB and BB were 0.64 and 0.77 min, respectively.

The 'fit statistics' in Table 4 denote less difference between the log-linear and Weibull methods for SimBB data at 60 and 61°C (with corresponding MSE and R^2 values which show no significant differences between the 2 methods). The concave downward response for cells in both PEP and BB was more accurately fit by the Weibull method. The most significant fit differences between the log-linear and Weibull methods can be seen for inactivation in PEP at all process temperatures (MSE values for the Weibull method were more than 50% lower than those for the log-linear method and R^2 values were >4% higher, denoting a more accurate fit by the Weibull method). It should be noted that R^2 values for both the linear and Weibull methods were >0.9 in all cases. z-

value estimates (the temperature change required to cause a one log cycle (90%) change in the log D-value) were calculated in this study (based on the 3°C range of process temperatures) and are also given in Table 4.

A functional comparison of the log-linear and Weibull methods was shown by the amount of time required for a specific decimal (log) reduction (Table 5). The Weibull method showed the need for longer processing times (compared to the log-linear method) for every combination of heating menstruum and process temperature. 4 log reduction times showed the largest differences between the 2 methods (with %Diff for Weibull time-to-reduction values >40% higher than log-linear values in some cases). For a 7 log reduction in all menstrua, the Weibull method was increasingly conservative (with respect to process time required) with increasing process temperature.

The differences in cell response in laboratory diluents (PEP and PB59) compared to beef broth menstrua (SimBB and BB) in this experiment could suggest a ground beef constituent effect (specifically, those constituents found in the free liquid filtrate of the ground beef). Stringer and others (*11*) compiled inactivation data for *E. coli* O157:H7 processed in a number of different heating menstrua and reported D and z-value estimates. Results for various meat products showed an average D_{60C} of 1.8 min (R^2 of 0.85) and a z-value of 5.5°C. These values were comparable to results found in this study for BB (D_{60C} of 1.84 min with an R^2 of 0.938 and a z-value estimate of 5.3°C).

Williams and Ingham (*14*) studied the effects of heat shock on the thermal resistance of *E. coli* O157:H7 in both laboratory media and a ground beef slurry. The ground beef slurry consisted of a 1:10 homogenate of irradiated ground beef in sterile

0.1% (w/v) peptone diluent. The researchers stated that the protective effects of ground beef constituents could possibly overshadow the protective effects related to heat shock, but they did not define the constituents. The researchers reported significant increases in D-value for heat shocked cells (held at 45°C for 10 min) inactivated in tryptic soy broth (with D_{58C} values of 2.2 vs. 3.7 min for non-heat shocked and heat shocked cells, respectively). This heat shock effect did not result in a significant change in heat resistance for cells processed in ground beef slurry (D_{58C} values of 4.2 and 4.1 min for non-heat shocked vs. heat shocked cells, respectively).

Ground beef constituents (as simulated in this study) do appear to have an effect on thermal inactivation of *E. coli* O157:H7. Results suggest that a simulated beef broth system (SimBB) could provide similar information to the free liquid filtrate of raw ground beef (BB) related to the thermal inactivation characteristics of *E. coli* O157:H7. One advantage of the SimBB method is that it is less laborious to prepare and more adaptable for experimental methodology. For the temperature range studied in this experiment, D-values for cells in SimBB were either more conservative (60°C) or not significantly different (61°C & 62°C) than those in BB. There were significant differences in response when cells were processed in laboratory diluent (PEP and PB59) compared to either the simulated or actual beef broth mixtures. The use of the Weibull model offered more flexibility and accuracy of fit over the traditionally used log-linear approach in this study. The use of the Weibull model was shown to estimate longer time-to-reduction values (for both 4D and 7D reductions) when compared with the log-

linear method. Further research to optimize nutrient concentrations and test a larger temperature interval would add significantly to this research.

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Appendix

Table 4 Model parameters and 'fit statistics' for log-linear and Weibull methods

Temp °C	n[1]	D-value min	z-value °C	δ[2]	p[3]	Linear MSE[4]	Weibull MSE	Linear R^2	Weibull R^2
				Peptone heating (PEP)					
60	52	1.45	4.19	3.45	1.76	0.412	0.155	0.927	0.971
61	52	0.85	4.19	2.38	1.88	0.295	0.049	0.940	0.990
62	36	0.48	4.19	1.57	2.12	0.172	0.037	0.953	0.990
				Phosphate buffer (PB59)					
60	30	1.93	3.48	3.32	1.33	0.072	0.035	0.986	0.993
61	30	0.91	3.48	2.28	1.67	0.358	0.155	0.943	0.975
62	19	0.51	3.48	1.77	2.15	0.135	0.139	0.966	0.969
				Simulated beef broth (SimBB)					
60	80	2.12	3.86	3.37	1.28	0.157	0.115	0.972	0.980
61	62	1.10	3.86	1.71	1.23	0.195	0.175	0.963	0.966
62	47	0.64	3.86	1.79	1.76	0.144	0.105	0.975	0.982
				Real beef broth (BB)					
60	51	1.84	5.30	3.84	1.59	0.263	0.144	0.938	0.966
61	71	1.13	5.30	2.02	1.34	0.077	0.064	0.984	0.987
62	48	0.77	5.30	2.16	1.84	0.221	0.099	0.962	0.982

[1]n represents the number of observations.
[2]δ is the Weibull hazard function parameter.
[3]p is the Weibull shape factor.
[4]MSE is the mean sum of squared errors.

Table 5 Time-to-reduction comparison (log-linear vs. Weibull methods)

Temp ºC	4-D[1] min	Weibull (4d[2]) min	7-D min	Weibull (7d) min	% Diff[3] 4-D	% Diff 7-D
			Peptone heating (PEP)			
60	5.79	7.59	10.13	10.43	33	3
61	3.40	4.97	5.95	6.70	20	13
62	1.92	3.02	3.36	3.93	11	17
			Phosphate buffer (PB59)			
60	7.72	9.41	13.51	14.34	44	6
61	3.64	5.23	6.37	7.31	22	15
62	2.06	3.37	3.60	4.38	7	22
			Simulated beef broth (SimBB)			
60	8.48	9.95	14.84	15.40	49	4
61	4.40	5.29	7.70	8.36	45	9
62	2.57	3.94	4.50	5.43	14	21
			Real beef broth (BB)			
60	7.36	9.18	12.87	13.05	40	1
61	4.54	5.68	7.94	8.62	40	9
62	3.09	4.58	5.41	6.21	18	15

[1]D represents D-value and is preceded by a multiplier of 4 or 7.
[2]d represents the decimal reduction for the Weibull method and is preceded by a multiplier of 4 or 7.
[3]The % Difference is based on Weibull versus log-linear method for an equivalent log reduction.

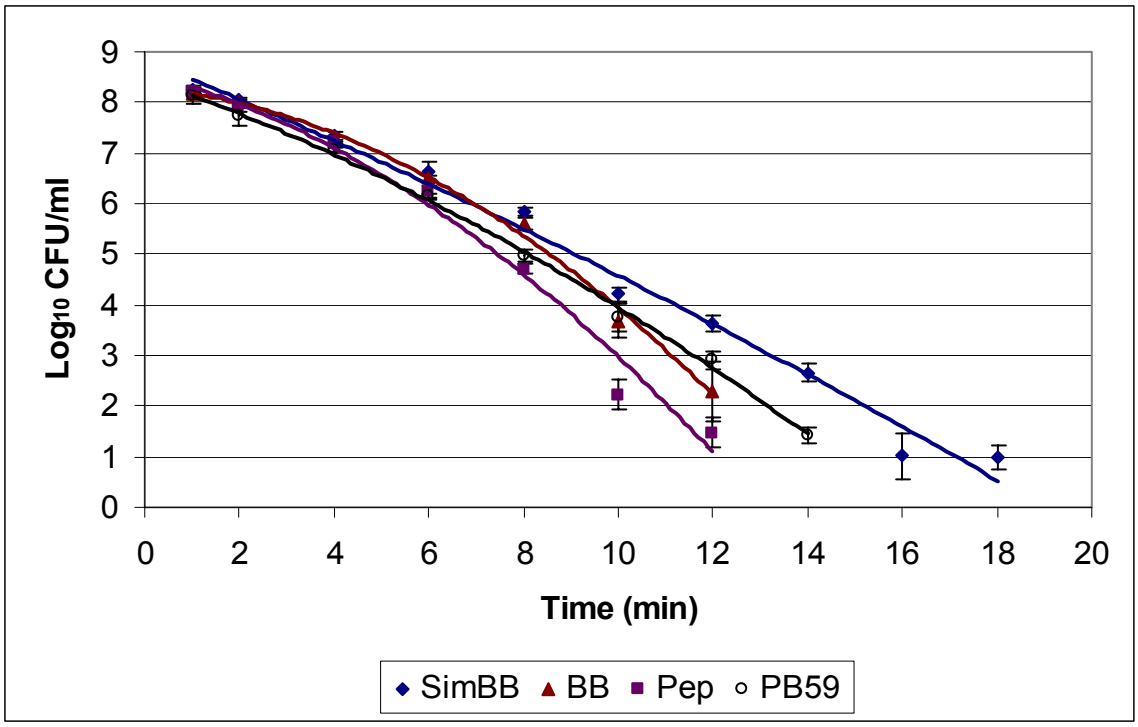


Figure 14 Inactivation Comparison of *Escherichia coli* O157:H7 at 60°C, in peptone (Pep), phosphate buffer (PB59), simulated beef broth (SimBB), and actual beef broth (BB) using the Weibull model (GInaFiT)

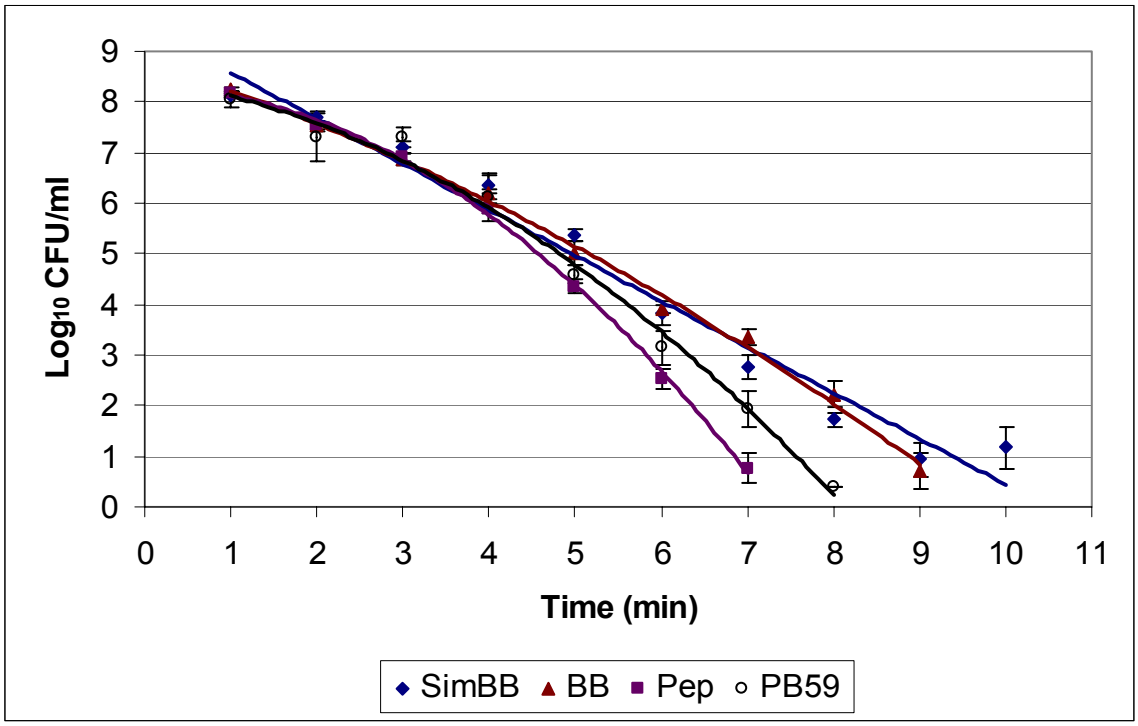


Figure 15 Inactivation Comparison of *Escherichia coli* O157:H7 at 61°C, in peptone (Pep), phosphate buffer (PB59), simulated beef broth (SimBB), and actual beef broth (BB) using the Weibull model (GInaFiT)

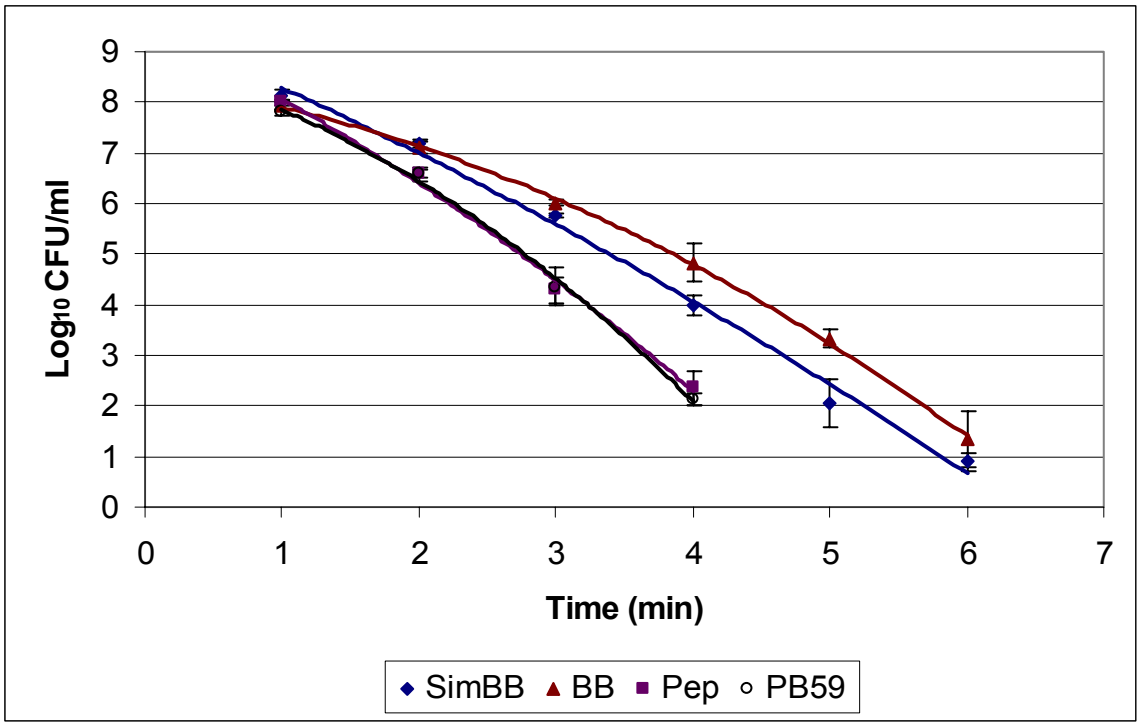
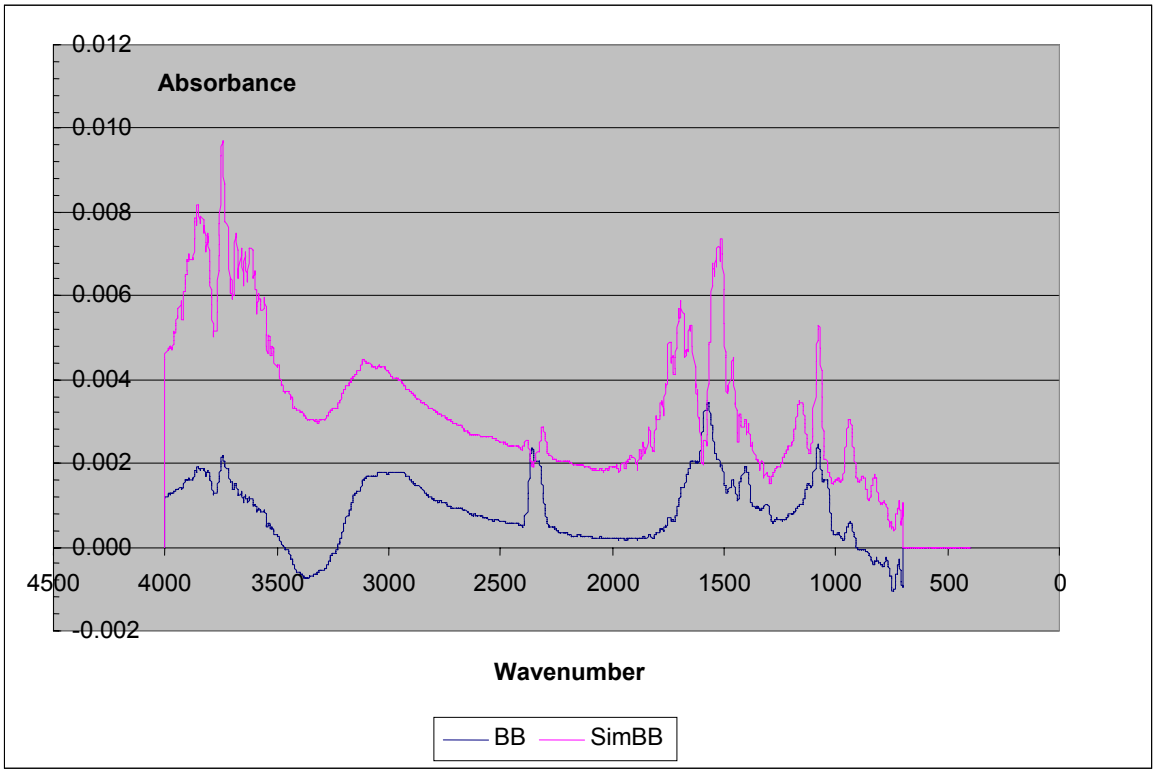


Figure 16 Inactivation Comparison of *Escherichia coli* O157:H7 at 62°C, in peptone (Pep), phosphate buffer (PB59, pH ca. 5.9), simulated beef broth (SimBB), and actual beef broth (BB) using the Weibull model (GInaFiT)

Fourier transform infrared (FTIR) measurements of the heating menstrua were performed using FTIR with attenuated total reflection infrared (ATR) accessory (Nexus 680, Thermo Nicolet Corp, Madison, WI) in the wavenumber range of 700-4000 cm-1 with resolution 4 and scan number of 64. Every sample was analyzed three times to generate a statistic average spectrum. The averaged spectra were smoothed using Savitsky-Golay algorithm. The number of points used in the smoothing process was 25. Results show similar peaks (relative to specific bonds and components) with some differences in absorbance (concentration). The SimBB formula represents the most accurate comparison to the actual BB.



PART FIVE: CONCLUDING REMARKS

I. Concluding Remarks

Numerous researchers have conducted experiments which model the survival or inactivation of specific food pathogens in either real food systems or laboratory media. Factors affecting microbial heat resistance, such as environmental conditions and intrinsic conditions specific to the microorganism, influence the design of the thermal process. The choice of appropriate regression and analysis tools will greatly affect the accuracy of applied modeling techniques and may offer more detailed characterization of underlying physiological response for well defined microbial inactivation studies.

Results from research conducted in this study suggest there are significant differences in the inactivation response for *Escherichia coli* K12 and O157:H7 depending on the physiological state of the cells. Environmental conditions, such as pH and heating menstruum, significantly influenced the overall thermal resistance for fixed temperature levels, while prior growth conditions of the cells influenced the shape of inactivation response.

Future work is needed to research additional environmental factors, in addition to and in combination with the ones explored in this study, and the effect of microbial growth conditions. Continued work to better understand and model the response of food pathogens under more realistic conditions is crucial to preserve the safety of the food supply.

Vita

Darryl (Glenn) Black was born in Greenwood, South Carolina on December 3, 1965. He was raised in Saluda County (SC) on his fathers' dairy farm and attended grade school, middle school and high school in the town of Saluda, SC. He graduated high school in 1984. Glenn joined the South Carolina Army National Guard in 1982 (honorable discharge in 1988) and completed basic training between his junior and senior years of high school. He attended Clemson University in Clemson, SC and received his B.S. in Agricultural Engineering in 1988 and an M.S. in Agricultural Engineering in 1994. Glenn worked for Pepsi Cola Company prior to completion of his M.S. (1991 to 1993). He worked as a project engineer for The Facility Group in Smyrna, GA from 1994 to 1995. He worked for Stork Food Machinery in Gainesville, GA from 1995 to 1998. Glenn worked outside the food industry for two and one-half years prior to working for Slim Fast Foods from 2001 to 2004 in Covington, TN.

Glenn will obtain his doctorate degree in Food Science and Technology, major Food Microbiology at the University of Tennessee, Knoxville, in May 2008. He is a member of the Institute of Food Technologists, International Association of Food Protection and Institute for Thermal Process Specialists. Currently he works as the "Director of Processing Technologies" for the Grocery Manufacturers Association in Washington, D.C.